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THE UNIVERSITY OF ALBERTA

Uptake, elimination and tainting involving benzothiophene and dibenzothiophene by rainbow trout (*Salmo gairdneri*)

BY

Edel M. Dromey

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE
DEGREE OF MASTER OF SCIENCE

IN

ENVIRONMENTAL SCIENCE

DEPARTMENT OF CIVIL ENGINEERING

EDMONTON, ALBERTA

(FALL, 1987)

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Uptake, Elimination and Tainting Involving Benzothiophene and Dibenzothiophene by Rainbow Trout (*Salmo gairdneri*), submitted by Edel Mary Dromey in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE, in Environmental Science.

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Supervisor

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Date: Oct 2/87

Abstract

Aquatic species in the nearshore environment may be exposed to petroleum derived pollutants. Certain of these pollutants may be bio-accumulated by the organisms, and can often impart an offensive odour to the fish flesh. Rainbow trout (*Salmo gairdneri*) were exposed to benzothiophene and dibenzothiophene, to determine if they could readily accumulate and depurate these chemicals; and to look for evidence of metabolism. Muscle, liver, and bile samples were extracted in the laboratory and analysed for parent compound by gas chromatography, using both flame ionization and flame photometric detectors. Extracts were further derivitized in the search for metabolites. In addition rainbow trout were exposed to varying concentrations of benzothiophene and a sensory evaluation was performed on the resulting flesh. Sensory experiments were focused on benzothiophene as it was suspected of having a low odour threshold in fish. Exposed flesh was compared with fortified flesh, to ascertain if there was a difference between the two.

The results indicate that benzothiophene and dibenzothiophene are readily bioconcentrated by rainbow trout, with the greatest accumulation being found in the liver. Exposure/depuration experiments demonstrated that after 65h depuration, significant amounts of both chemicals could still be found in the tissues examined. Although no metabolites of either compound were found with the methods employed, further research is needed in order to confirm or deny the lack of metabolites.

Experiments with the sensory panel confirmed that: (i) benzothiophene had a very low detectable threshold for exposed fish and (ii) panelists had a preference for exposed over fortified flesh. Fish exposed to benzothiophene were found to be significantly more tainted than those fortified with the chemical. The median odour

threshold value was found to be 0.01 mg/kg. This was the lowest concentration presented to the panelists, therefore it is suspected that the actual odour threshold could be lower.

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1. Introduction and Problem Statement

Oil discharged to rivers can have deleterious effects upon aquatic organisms. Of particular concern is that portion of oil which is soluble in water. The concentration and composition of hydrocarbons in the aqueous phase is different from the parent oil as more soluble components, such as aromatics, are concentrated there. This fraction can achieve intimate contact with fish and other pelagic organisms and it is not removed by gravity oil separation devices (Birkholz, *et al.* , 1987).

In January, 1982, a fire and series of equipment failures at the Suncor oil sands plant, north of Ft. McMurray, Alberta resulted in a spill of coker distillate fractions (thermally cracked bitumen, which has not been completely hydrotreated to remove sulphur and nitrogen compounds) to the plant's wastewater pond. Over the subsequent 10 week period, plant records reported the release of more than 50 tons of oil and grease into the Athabasca River under winter ice cover.

Subsequent analysis on fish (walleye) taken from the Athabasca River, by Wellington Environmental (1983, cited in Hrudey and Nelson, 1986) indicated the presence of aliphatics, polycyclic aromatic hydrocarbons (PAH) and polycyclic aromatic sulphur hydrocarbons (PASH) in the fish flesh. These contaminants can pose environmental problems at very low concentrations. Bioaccumulation can lead to much higher concentrations of some contaminants in fish tissue than found in water (Eastmond *et al.* , 1984). Many of these contaminants cause taste and odour problems in water at extremely low concentrations (Zoeteman *et al.* , 1971).

Birkholz *et al.* (1987) characterized the spilled oil from the Suncor plant. Alkylated benzenes, alkylated benzothiophenes and alkylated dibenzothiophenes were identified and

confirmed. Studies have shown that the PASH may be the most persistent of the polycyclic aromatic hydrocarbons in the environment (Lee *et al.*, 1972) and they have been found to bioconcentrate in fish and shellfish (Ogata *et al.* , 1977; Ogata and Miyake, 1980; and Ogata and Fujisawa, 1983).

Additional work by Wellington Environmental matched specific compounds found in the tissue with those identified in the Suncor waste oil. Although PAH and PASH were contained in the tainted fish from the Athabasca River, direct correspondence between identified compounds and those primarily responsible for the sensory taint was not established. There is very little known about which contaminants and what quantities are necessary to impart an offensive odour/taste to fish, or indeed to what extent they bioconcentrate. The PASH have been labelled as a possible major source of tainting in fish (Connell and Miller, 1981) but there is no experimental evidence to prove this. Consequently, there is a need to explore the nature of these contaminants which arise from the petroleum industry.

This particular research was designed to determine:

* if certain PASH, namely benzothiophene and dibenzothiophene readily accumulate in fish tissue.
* if fish can readily depurate these compounds after exposure.
* at what level benzothiophene imparts an offensive odour (taint) to the fish.
* if there is any sensory difference between fish flesh from fish exposed to benzothiophene in water and those spiked with benzothiophene after processing.
* if there is any evidence of metabolism for either of these chemicals.

2. Literature Review

2.1 Oil Tainting Problem

2.1.1 Introduction

Tainting of commercial species of fish crustaceans, oysters and muscles by petroleum products has been extensively reported in the literature (Fetterolf, 1964; Connell, 1974; Connell and Miller, 1980; Motohiro, 1983; and Hrudey and Nelson, 1986). Most of the tainting in the flesh of fish is due to the activities of the petroleum and chemical industries (Nitta, 1970, cited in Motohiro, 1974). Connell and Miller (1981) report that the taint which is associated with petroleum in aquatic species is due to an extremely complex mixture of substances associated with petroleum oil. They suggest that if fish ingest petroleum products, there is a possibility that tainting will occur. In addition, low levels of dissolved petroleum hydrocarbons in the water soluble fraction can be absorbed into muscle tissue of aquatic organisms and impart a taint to them. Thus tainting can occur through adsorption of dissolved components from the water through the gills (Lee, *et al.*, 1972, 1976; Varanasi, *et al.*, 1979; Roubal, *et al.*, 1977 and Sanborn and Malins, 1977) and adsorption of the dissolved component through the skin (Varanasi, *et al.*, 1978).

Although there are numerous references on the occurrence of tainting due to petroleum and petroleum products, there is little reported on the specific causative compounds. Stansby (1978) reports this area of research as being most neglected and recommends further research. Motohiro (1983) corroborates this view, suggesting that additional research is required on the behavior *in vivo* and the biochemical reaction between petroleum compounds and living tissues *in vitro* , with reference to tainting of fish flesh.

Connell and Miller (1981) report high sensitivity of the olfactory system to sulphur compounds. They suggest that such minor components of a petroleum substance may be major contributors to its aroma. Hrudey and Nelson (1986) reported that tainted fish from the Athabasca River contained both PAH and PASH, but the causative compound(s) primarily responsible for the taint was not identified. While there are few reports in the literature on specific PAH imparting an offensive odour to fish, there are even less (if any) on PASH. It is for this reason that PASH are specifically dealt with in this report.

2.2 Sources of PASH in the Environment:

2.2.1 PASH in petroleum and petroleum products:

Crude petroleum and most refined petroleum products are extremely complex mixtures of thousands of organic compounds (Neff, 1979). Hydrocarbons are most abundant, usually representing more than 75% of the weight of the oil (Jones *et al.*, 1977). The remainder is made up primarily of various sulphur, nitrogen and oxygen containing organic compounds. PASH include such compounds as benzothiophene and dibenzothiophene. Unfortunately, PASH comprises an incredibly complex mixture of individual specific compounds. For example Nishioka *et. al* (1986) presented structures for 62 different sulphur heterocycles (presented in Figure 1) which could occur in petroleum. This list did not include any of the many alkyl substituted components which occur. If the latter, which are known to exist in petroleum, were considered, the total number of possible PASH compounds becomes very large.

The composition of petroleum products is dependent upon the raw source (crude oil, coal, shale oil, etc.) the degree of chemical modification (such as cracking) and the physical separation method employed. Coleman *et al.* (1973, 1984) studied the

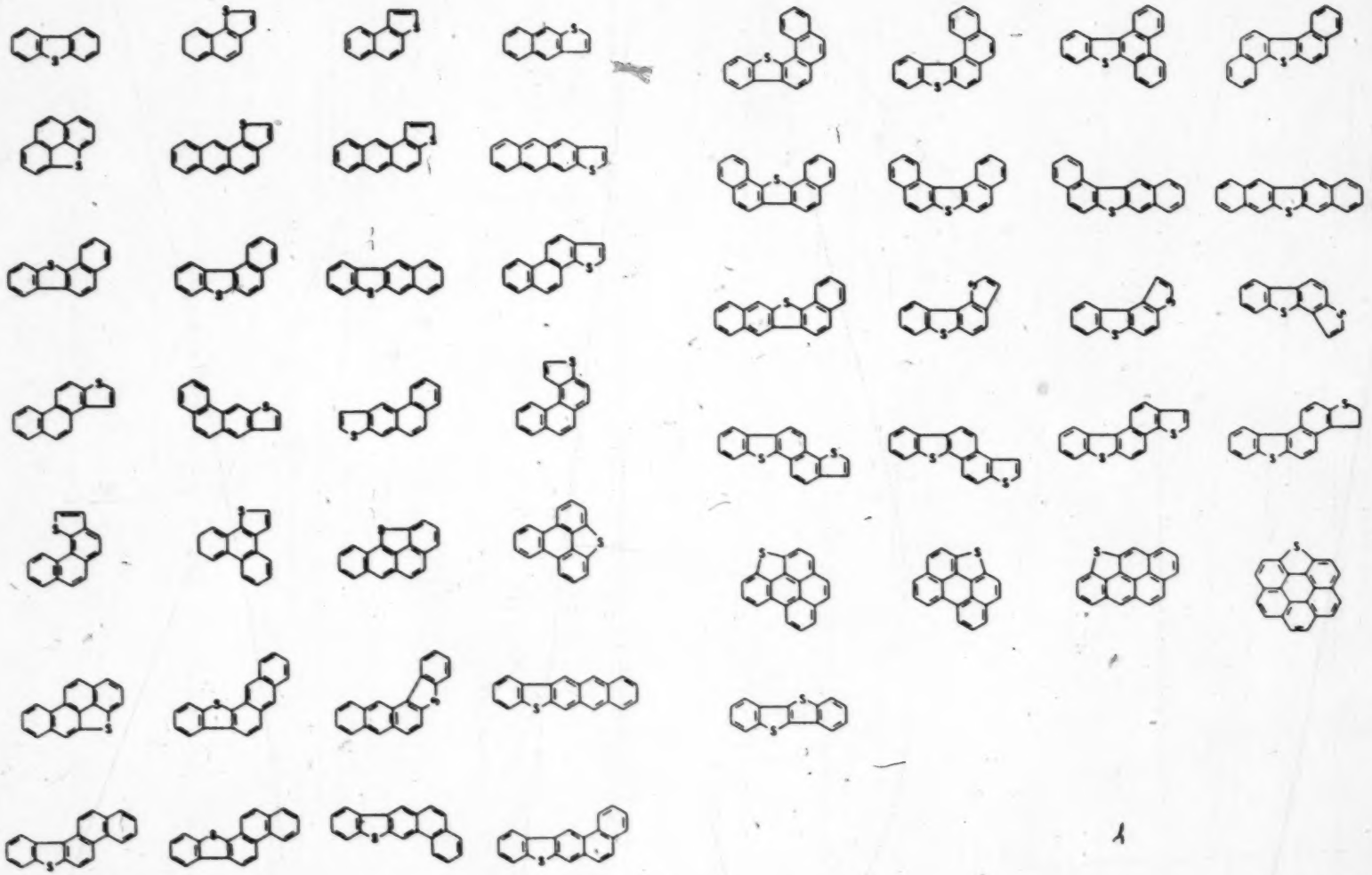


Figure 1. Example of possible sulphur heterocycle structures.
(Adapted from Nishioka, et al., 1986).

composition of the water soluble fractions (WSF) of certain petroleum products namely: gasoline, kerosene and No. 2 Fuel oil. The WSF was chosen in order to answer the question as to which components are likely to enter the drinking water supplies. It was shown that for all three fuels, aromatics comprised 50% or less of the weight of the product when analyzed directly. However, in each case aromatics comprised >93% by weight of the water-soluble fraction. Of the total aromatic content, PASH were found to represent between 3 and 15% by weight.

2.2.2 PASH in coal:

The organic matter in coal consists largely of a macromolecular material of complex and variable composition. A large number of complex PAH and heteroaromatic compounds are formed when coal is pyrolyzed (Eaton *et al.*, 1978; and Poston & Purdy, 1986). However, it is difficult to relate the products formed in this way to the original structure of coal. According to Neff (1979), 70 to 75% of the carbon present in bituminous coal is aromatic. PASH may comprise from 0.2 to 12% of the total aromatic carbon content.

2.2.3 PASH in bitumen:

Selucky *et al.* (1977) performed a detailed analysis on Athabasca bitumen. The bitumen analysed was separated into two groups: saturates and unsaturates. The latter was separated into mono-, di- and polyunsaturates. The polyaromatics (which contain the PASH series) were found to contain 7.07% by weigh sulphur compounds. The mono- and di- aromatics were found to contain 0.26 and 2.94% sulphur content by weight. Mass

spectral analysis of the polyaromatic fraction suggested that about 12% of the total sulphur content was of the benzothiophene series.

2.2.4 PASH in bitumen (oil sands) wastewater:

Hrudey & Nelson (1986) found the data base on characterization of the oil sands wastewaters to be limited. Wastewater characterization indicated a variety of alkylated aromatics and heterocyclic compounds with substantial quantities of sulphur compounds. Birkholz *et al.* (1987) characterized the water soluble components of an oil sands wastewater obtained from the Suncor plant in Ft. McMurray. Aromatic compounds predominated with alkylated dibenzothiophenes being positively identified and confirmed. The results suggest that C_1-C_3 dibenzothiophenes are likely present in the water soluble fraction.

Strosher & Peak (1976) found that tailings pond dyke drainage from the Great Canadian Oil Sands (now Suncor) plant contained 100 to 120 mg/L of total organic carbon. The total organic carbon contained 69% extractable carbon, which was mainly oxygenated compounds (92%). These oxygenated compounds were found to contain 5.3% sulphur compounds. Within the upgrading plant wastewater, Strosher & Peake found an average of 36 mg/L of total organic carbon. This total organic carbon was found to contain 42% extractable carbon. The extractable carbon was found to contain 17% organic sulphur compounds. Among compounds tentatively identified in the upgrading plant wastewater were benzothiophenes and dibenzothiophenes.

At a later stage, Strosher and Peake (1979) found the upgrading plant wastewater contained 47% extractable carbon for an average of 36 mg/L of organic carbon. The extractable carbon was found to contain 24% organic sulphur compounds which were

mainly substituted benzothiophenes. Although this number differs from the 1976 findings, Hrudey & Nelson (1986) are not surprised at the scale of variation because of the large portion of organic matter which cannot be accounted for.

2.3 Research into PASH Related Tainting Problems

There have been no papers in the literature dealing with the uptake and subsequent taintability of specific PASH in fish species. Certain authors have demonstrated that fish bioaccumulate PASH (Eastmond *et al.*, 1984; and Ogata and Fujisawa, 1983) but they have made no connection between uptake and subsequent tainting.

Ogata *et al.* (1977) demonstrated that eels reared in crude oil accumulate organic sulphur compounds. Ogata and Miyake (1978, 1980) characterized these sulphur compounds as being alkylated benzothiophenes. Ogata and Fujisawa (1983) found accumulation of alkylated dibenzothiophenes (C_1 to C_3) to tissue concentrations of 6 and 9 ppb in oyster and mussel respectively. These correspond to bioconcentration factors from 1845 to 28500.

Motohiro (1983) cites Yoshida and Uezuieu (1961) and Kaneda and Yasumoto (1974) as reporting sulphur compounds to be found in tainted fish. Wellington (1983) reported PASH ranging from 30 to 1500 ppb in tainted Walleye from the Athabasca River.

Connell and Miller (1981) suggest sulphur compounds may be a major contributor to the aroma of petroleum. GESAMP (cited in Connell and Miller, 1981) report dibenzothiophenes as being one major tainting component, in addition to several other classes of compounds.

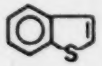
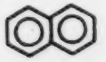
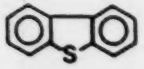
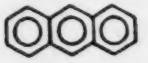
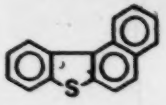
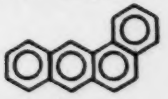
Paasivirta *et al.* (1981) and Sinkkonen (1982) measured a range of parts per million concentrations of alkyl dibenzothiophenes in fish and muscle from the Baltic. They found a good correlation between total oil residues and the alkyl dibenzothiophenes.

Vassilaros *et al.* (1982) analyzed catfish in the vicinity of a coking plant outfall for PAH and PASH. Parts per million concentrations of specific compounds were found from each class. The authors presented the argument that PASH would be of greater environmental significance than PAH, owing to their higher water solubilities. Structures, solubilities and octanol/water partition coefficients of the specific compounds are presented in Table 1.

Eastmond *et al.* (1984) compared a series of PASH and their sterically and structurally similar PAH for bioconcentration, elimination and toxicity in *Daphnia magna* . Benzothiophene was compared to naphthalene, dibenzothiophene with phenanthrene and benzo(b)naptha [2,1-d]thiophene with chrysene. Their results are summarized in Table 2. These suggest a greater degree of bioconcentration for the PASH than for the PAH. There was no obvious trend for observed elimination differences between PASH and PAH. The PASH were generally more toxic than their analogous PAH.

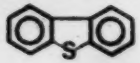
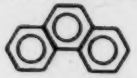
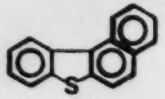
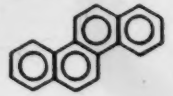
Vassilaros *et al.* (1982) and Vandermeulen (1981) cited in Eastmond *et al.* (1984), indicated that PASH associated with oil spills may be depurated more slowly than their PAH analogues and are more resistant to microbial degradation. There is no evidence in the literature of uptake and elimination of PASH by fish as related to tainting problems.

Table I Comparison of octanol/water partition coefficient and water solubility for Selected PASH and PAH[a]

PASH	Log P	Water Solubility	PAH	Log P	Water Solubility
thiophene	1.6	1430.	benzene	1.6	1515.
benzothiophene 	3.1	113.	naphthalene 	3.4	30.
dibenzothiophene 	4.4	1.7	anthracence 	4.4	0.04
benzo[*b*]naphtho-[1,2-*d*]thiophene 	5.6	0.06	benz[*a*]-anthracene 	5.6	0.01

[a]after Vassilaros et al.(1982)

Table 2 . Summary of peak bioconcentration factors and elimination half lives for PASH and PAH in *Daphnia magna*.[a]

PASH	Peak BCF	Elimination half-life (h)	PAH	Peak BCF	Elimination half-life (h)
benzo[*b*]thiophene	750	27	naphthalene	50	b
dibenzothiophene 	600	12	phenanthrene 	600	b
benzo[*b*]naphtho-[2,1-*d*]thiophene 	8000	23	chrysene 	5500	18

[a]after Eastmond et al. (1984)

[b]elimination not first order

2.4 Pathways of PASH in the Aquatic Environment

PASH may reach the aquatic environment by various routes including: combustion or spillage of fossil fuels; industrial and domestic wastewater discharges; runoff from land; and atmospheric fallout. Crude oil and distillate products entering the aquatic environment each year from material and anthropogenic sources was estimated by the National Academy of Sciences (1973, cited by Neff, 1979) to be approximately 6.1 million metric tons per year. Neff, assuming a mean concentration of 2.8% PAH in crude oil, estimated that 1.7 million tons of PAH are entering aquatic systems each year from all sources. Of course, PASH would be some fraction of that estimate.

Very little is known about the amounts of PASH entering the aquatic environment each year. Although estimates have been done for PAH, they cannot be reliably transferred to PASH.

Metabolism of PASH:

2.5.1 Introduction:

A detailed knowledge of the metabolism of PAH in aquatic species is essential to assessing the consequences of petroleum pollution. The mixed function oxygenase (MFO) system is an enzyme system which responds readily to the presence of foreign chemicals (Malins, 1977; Varanasi, 1979). There are numerous studies in the literature detailing the activation of the MFO's by PAHs, but there have been no studies on the metabolism of PASH.

Owing to a lack of such research , PAH analogues must be reviewed in order to obtain an indication of the possible metabolic pathways of the corresponding PASH. The limitations of such a comparison must be stressed: PASH do not necessarily follow the same pathways as PAH. For the purpose of this review, naphthalene has been chosen as the PAH analogue to benzothiophene.

2.5.2 Overview: enzyme systems governing the metabolism of aromatic hydrocarbons:

MFO enzymes, which are NADPH-dependent, catalyze the introduction of oxygen into the aromatic nuclei (Malins, 1979). This enzyme system is variously known as cytochrome P450 dependent MFO, oxygenases, aryl hydrocarbon hydroxylases (AHH) or drug metabolizing system. The MFO system is located in the liver and sometimes in other organs such as the spleen (Neff, 1979). Within the cell, it is localized primarily in association with the endoplasmic reticulum (Andersson and Forlin, 1985) and consists of several components, including a nicotinamide adenine dinucleotide phosphate (NADPH) cytochrome reductase, a phospholipid and the heme protein called cytochrome P448 or P450. These components function as an electron transport system when combined with NADPH as a source of reducing power, molecular oxygen and an appropriate substrate such as PAH (Neff, 1979). Neff reports that such a system catalyzes the incorporation of one atom of molecular oxygen into water and one atom into the substrate. The general reaction involved is as follows:

$$RH + NADPH + O_2 + H^+ \longrightarrow NADP^+ + H_2O + ROH$$

The oxygenases are believed to account for the formation of virtually all the primary metabolic products of aromatic hydrocarbon degradation (Von Hofe & Pufer, 1986). The presence of this xenobiotic biotransformation enzyme system in fish is now well established (Lech, 1973, Lech and Bend, 1980, Andersson and Forlin, 1985 and Oikari and Kunnamo-Ojalo, 1987). Recently, electrophilic intermediates (such as the epoxide group) in the formation of the hydroxy derivatives, have been viewed with interest (Andersson and Forlin, 1985; and Oikari and Kunnamo-Ojalo, 1987). Reactions involving the conjugation of epoxides and phenols with water soluble substances give rise to mercapturic acid derivatives, glucuronides, sulphates and glycosides in liver and kidney (Lech and Bend, 1980 and Andersson & Forlin, 1985).

Collier *et al.*, (1978) and Bend *et al.* (1979) have shown that metabolites of several aromatic hydrocarbons excreted into the bile are hydroxylated and glucuronidated products, whereas sulphates are present in very low amounts, or absent. These reports indicate that foreign compounds such as BaP and naphthalene in fish are metabolized via sequential enzyme systems in the liver and that conjugated xenobiotics are the endproducts of xenobiotic biotransformation.

2.5.3 Metabolism of naphthalene

Studies with naphthalene may be of some help in trying to identify metabolites of PASH, as they may follow the same or similar pathways of metabolic degradation. However further research is required, specifically in the area of developing new analytical techniques for identifying PASH metabolites. Lee et. al (1972) studied the uptake and metabolism of 14C naphthalene in three species of marine fish. They found that the compound was metabolized in the liver and subsequently deposited in the bile: a major storage site of hydrocarbon metabolites. In all tissues the major compound after 48h of exposure was found to be 1,2-dihydro-1,2-dihydroxynaphthalene.

Malins *et. al* (1979) exposed *S.gairdneri* to radiolabeled naphthalene. Isolation of metabolites from the liver resulted in the identification of two non-conjugates (1-napthol and 1,2-dihydro-1,2-dihydroxynaphthalene) and three conjugates (1 napthyl glucuronic acid, 1-napthyl sulphate and 1-napthyl glucoside). Major components of the metabolite fractions were 1,2-dihydro-1,2-dihydroxynaphthalene and glucuronic acid derivitives.

Krahn *et. al* (1980) force fed rainbow trout gelatin capsules containing naphthalene. Metabolites were determined using HPLC/UVF. As such, no attempts were made to identify 1,2-dihydro-1,2-dihydroxynaphthalene since it does not fluorece strongly. The bile was examined and one non conjugate (1-napthol) and two conjugated metabolites (1-napthyl glucuronide and 1-napthyl sulphate) were identified.

Varanasi *et al.* (1978, 1979, 1982a and 1982b); and Varanasi and Gmur (1980, 1981) carried out numerous experiments on the metabolism of naphthalene in aquatic species. Varanasi *et al.* (1979) force fed juvenile starry flounder and rock sole naphthalene dissolved in salmon oil. They reported high concentrations of naphthalene and its metabolic products 24h after the force feeding commenced in starry flounder, with maximum concentrations being reached at 48h. Significantly greater amounts of naphthalene and its metabolites were found in rock sole.

In the bile of both fish, glucuronic acid conjugates were the major metabolites. Small amounts of mercapturic acid derivatives were present also. Non-conjugated metabolites, such as 1 and 2-naphthols and 1,2-dihydro-1,2 dihydroxynaphthalene constituted less than 10% of the total extracted metabolites. In the livers of both fish, 1,2-dihydro, 1,2-dihydroxynaphthalene was the major metabolite (38.7% for rock sole and 41.5% for starry flounder) at 24h after feeding. Considerable proportions (31.9%) of conjugates (eg. glucuronides, mercapturic acids, glucosides/sulphates) were also found in the liver of rock sole, whereas metabolites in starry flounder were mainly non-conjugated

(76.7%). However, with time, there was an increase of conjugated metabolites found in the liver. Varanasi & Gmur (1981) found that glucuronides were the major metabolites of naphthalene in liver of English sole. On hydrolysing these glucuronides the authors revealed that a large proportion (70%) was the 1,2-dihydro-1,2-dihydroxynaphthalene. This was the major metabolite found in the liver of rock sole in the preceeding study, by Varanasi *et al.* (1979).

Varanasi *et al.* (1978) studied the uptake and release of naphthalene and its metabolites through the skin of fish. The skin is a primary site of contact with environmental pollutants and had been largely overlooked in studies concerning the fate of petroleum in aquatic organisms. Rainbow trout were exposed to naphthalene through: force feeding, via injection; and water immersion studies. The authors found that regardless of mode of exposure, the skin accumulated significant concentrations of naphthalene and its metabolites. The results also indicated that, similar to the liver, the skin has the ability to preferentially retain metabolites.

3. Materials and Methods

3.1 Materials:

Benzothiophene and dibenzothiophene were obtained from Fisher Scientific Company. Purity was confirmed to be 98% using GC/FID and GC/MS. Celite, florisil, sodium bicarbonate (solid), acetic anhydride (97%), anhydrous sodium sulfate, phosphoric acid (85%), ammonium hydroxide (30%) and pesticide grade solvents (chloroform, methylene chloride, methanol and hexane) were also obtained from Fisher Scientific. The anhydrous sodium sulphate and celite were purified by soxhlet extraction using methylene chloride for 12 hours. Following extraction, the solvent was evaporated in a vacuum fume hood and the materials then stored in a convection oven maintained at 130 ^{o}C, until required.

Ammonium phosphate buffer (pH 6.5) was prepared by adding 1.0 mL of phosphoric acid to 1 L of organic free water and titrating to pH 6.5 by dropwise addition of ammonium hydroxide (30%). Potassium phosphate buffer (pH 7.0) was prepared by titrating 50 mL of 0.1 M potassium dihydrogen orthophosphate (obtained from Fisher Scientific) with 0.1 M sodium hydroxide (Fisher grade) to pH 7.0. Acetate buffer (0.2M) was prepared titrating 50 mL of 0.2 M acetic acid with 0.2 M sodium acetate to pH 5.0. Acetate buffer (2.0 M) was prepared by titrating 50 mL of 2.0 M acetic acid with 2.0 M sodium acetate to pH 5.0.

a-Napthyl-b-D-glucuronic acid and glucuronidase (containing sulphatase activity) were obtained from Sigma chemical company. The b-glucuronidase solution (Sigma, G.8132) was prepared by dissolving 10-12 mg of the enzyme in 500 mL of 0.2 M acetate buffer. 100 mL of this solution gave approximately 2000 units of enzymatic activity.

Organic free water was obtained by passing distilled water through a trace organic removal cartridge (Millipore Corp.).

Rainbow trout were obtained from the Alberta Environmental Centre, Vegreville, Alberta. They averaged 150-300 g in weight and 200-300mm in length. The fish were held in a 1400 L fibreglass tank, at an average temperature of 11 °C. The tank was aerated with the aid of three aquarium size air diffuser stones. While in the holding tank, the trout were fed a diet of pellet food. A diurnal cycle of 8 h light and 16 h dark was observed throughout the experiments.

3.2 Equipment:

Gas chromatography was performed using a Varian (model 3500) GC and a Hewlett-Packard (model 5880) GC. The Varian 3500 instrument was equipped with a flame photometric detector, a model 1077 Grob type split/splitless injector (operated in the splitless mode) and a model 600 data system. The column used was a 30 m X 0.32 mm (i.d.) fused silica, wall-coated DB-5, obtained from J&W Scientific Inc.. The carrier gas was helium and the temperature increased from 40 to 280 °C at 10 °C/min., beginning 1 min. after injection. The oven temp was maintained at 280 °C for 10 min. and the detector temperature maintained at 300 °C.

The H-P model 5880 was equipped with a model 1883B Grob type split/splitless injector system (operated in the splitless mode), flame ionization detector, model 7672A autosampler and level four data processing capability. It contained a 30 m X 0.32 mm i.d. fused silica, wall-coated, DB-1301 capillary column, obtained from J&W Scientific Inc.. The carrier gas was helium and the temperature increased from 40 to 280 °C at 10 °C/min., beginning 1 min. after injection. The oven temperature was maintained at 280 °C for 20

min. and the injector and detector temperature were maintained at 270 °C and 300 °C respectively.

Substances were confirmed using a HP model 5970 quadrupole mass spectrometer interfaced with a HP model 5980 GC. This GC was equipped with a model 1883B Grob type split/splitless injector (operated in the splitless mode) and a 12.5 m X 0.2 mm (i.d.) fused silica, wall-coated, HP-1 capillary column. The carrier gas was helium and the temperature increased from 40 to 300 °C at 10 °C/min. beginning 1 min. after injection. The oven temperature was maintained at 300°C for 8 min. and the injector maintained at 250 °C. The data system was a HP model 5997, with data acquisition beginning 2 min. after injection. Mass spectral scans were obtained every 1.36 seconds.

The HPLC (High Performance Liquid Chromatograph) Waters Chromatography Division system consisted of the following units: two model 6000A pumps, a Waters automated gradient controller, a model 680 solvent programmer, a model 710B autosampler, a model 450 variable wavelength detector and a Hewlett-Packard model 3388A integrator for recording and integrating chromatograms. Separations were performed using a 15 cm X 4.6 mm i.d. Supelcosil LC-PAH column, frit size 5 µm (Supelco Inc.). Analysis was performed in the isocratic mode using a solution of acetonitrile (70%) and ammonium phosphate buffer, pH 6.5 (30%). The flow rate was maintained at 3.0 mL/min. and absorbance was set at 233 nm.

The soxhlet extraction apparatus consisted of a soxhlet extractor, Friedrich condensor and a 300 mL round bottomed flask obtained from Kontes Scientific Glassware Inc. Extraction thimbles (45 mm X 130 mm frit, porosity 40 µ) were obtained from John Scientific Inc. The six soxhlet extractors were mounted in an electrothermal extraction

assembly (Fisher Scientific) which consisted of electrothermal heaters and the necessary support apparatus to mount the soxhlet apparatus.

The nitrogen evaporator used was a Meyer Organomation model III. The water bath employed with this evaporator unit was maintained at 50°C. The electric shaker used was a Burnett, model 75 wrist action shaker. The polytron was obtained from Brinkman instruments, a Kinematica type PT10-35. The centrifuge employed was a model CL obtained from International Equipment Co (I.E.C.).

3.3 Fish Exposure Facilities and Procedures:

3.3.1 Initial Studies:

Fish exposure methodologies were initially assessed in the Department of Zoology, University of Alberta. The response of rainbow trout to various different parameters was quantified.

Fish of similar size in the same aquaria were compatible. However, when a large and a small fish were placed together, aggressive behavior was observed. The smaller fish was unable to protect itself and incurred substantial superficial injuries as a result. Thus in addition to allowing 1 L of water for every 5- 7 g of fish weight, similar sized fish were always placed together in the same aquarium, to reduce this stress associated with the aggressive behavior.

In these preliminary studies, oxygen levels in non-aerated tanks were carefully monitored and the response of rainbow trout noted. Fish became visibly stressed around 4.7 mg/L dissolved oxygen and below 3.7 - 4.0 mg/L death was imminent. Dissolved oxygen levels, therefore, were never allowed to drop below 4.0 mg/L at any time during

the exposure experiments. Although this level was reached on two occasions only, the water was re-aerated to bring D.O. levels back up to 7.0 mg/L or better. A water temperature of 12 °C was maintained in the exposure tanks and appeared to be an adequate temperature for the trout as well.

Little was reported in the literature regarding the toxicity of PASH to fish. Therefore, LC_{50}'s for benzothiophene and dibenzothiophene were determined and estimated as 8.0 and 5 mg/L respectively. The experimental method for determining the LC50's consisted of exposing a number of fish (6 fish with 2 per tank) to varying concentrations of the chemical, ranging from 0.01 mg/L to 10 mg/L. The LC50 was defined as that concentration where >50% responded (died) in a specified time period (8 h). From these experiments a concentration of 1 mg/L was chosen for exposure and exposure/depuration studies for both the benzothiophene and dibenzothiophene. This choice was based on the LC50 findings: 1 mg/L would not likely kill the trout for the exposure period contemplated.

Trial experiments were also run to determine the extent of loss of the chemicals from the exposure tank due to factors such as volatilization and adsorption to the liner bags. In order to obtain the desired concentration in water, it was necessary to add the chemicals dissolved in water soluble solvents; methanol for benzothiophene and acetone for dibenzothiophene.

Logistical problems, such as how to add the chemicals to the water, were also worked out at this time. When added directly with no solvent and no mixing, very low concentrations of chemicals were found in the water. However, with the aid of a hand-held rotary mixer and the carrier solvents mentioned above, the desired concentrations could be obtained during the initial phases of the experiments at least.

Overall the initial experiments proved valuable in the development of the exposure methodology. As a result, the actual exposure/depuration studies were run smoothly and in the minimum of time.

3.3.2 Main Holding Tank

A 1400 L circular fibre glass tank was used to maintain fish stocks for experimental purposes (Figure 2). Water within the tank was maintained through a combination of recirculation (with filtration and cooling) and fresh water input. Organic- and chlorine-free water was provided by running available tap water through two 1.2 m long X 10 cm diameter granular activated carbon columns. This treated water was allowed to flow into the holding tank at an average rate of 1.5 L/min.. An overflow was provided at the opposite end of the tank to allow drainage.

The tank water was continually cleaned and cooled through a recirculation system (Figure 2). Water was pumped from the bottom of the holding tank through a sediment trap (for large particle removal) followed by a fine mesh cloth filter (25 mm polyester) for a more thorough cleanup. The water was finally passed through a refrigeration unit (Coardley Broadview Model R2-10) and cooled to a temperature of 8-9 ^{o}C prior to re-entry (along with the fresh water) into the holding tank.

Water temperature, dissolved oxygen and pH were monitored continuously using a Yellow Springs Instrument(Y.S.I.), model 310 Dissolved oxygen/temperature meter and an Fisher model 805 MP pH/MV meter. Filtered, freshwater, was checked biweekly for free chlorine using the DPD (N,N-Diethyl-p-phenyl-enediamine) colourmetric method (

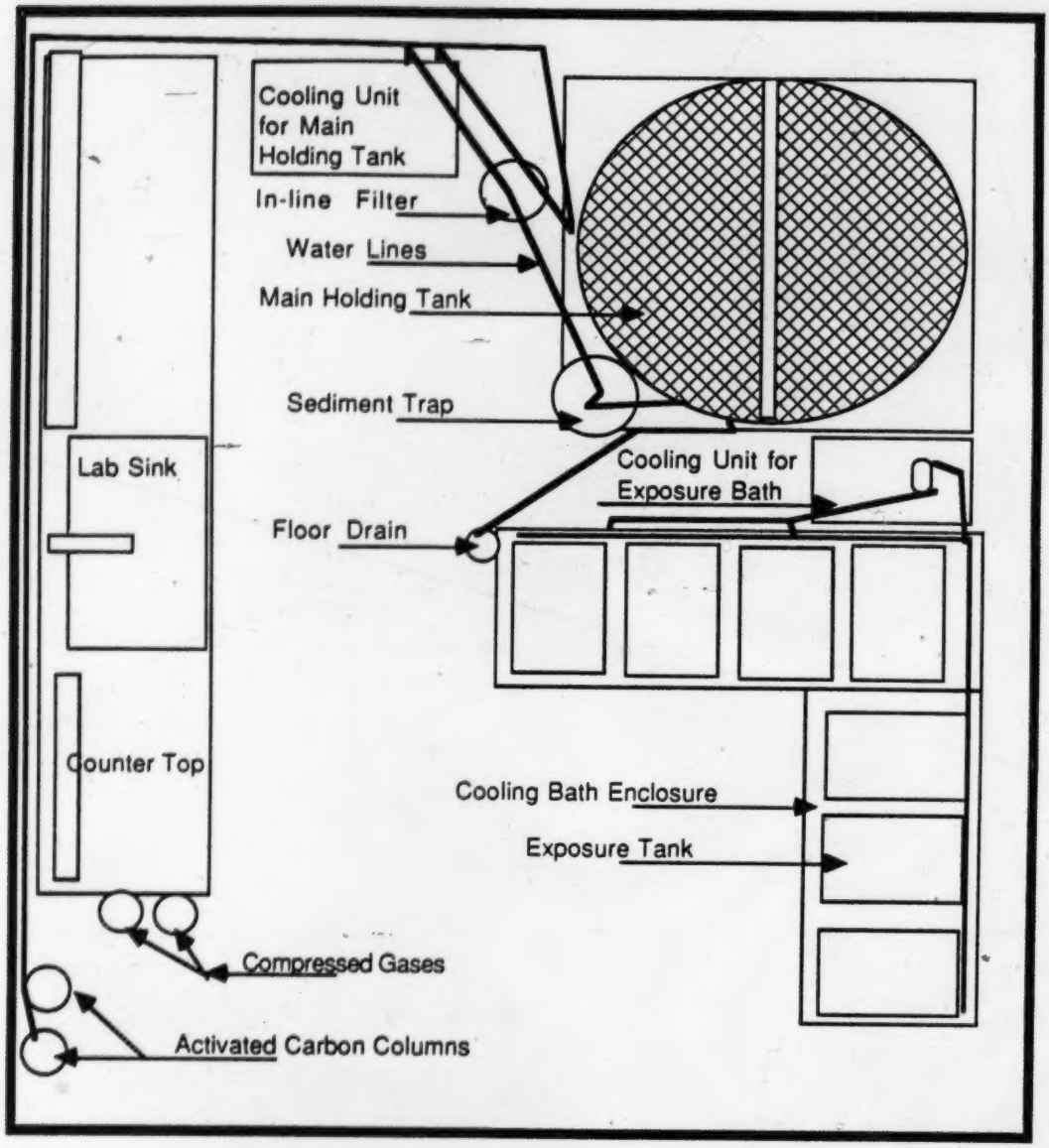


Figure 2. Schematic of fish holding facilities.

APHA, 1986). In addition, the filtration unit was removed and thoroughly cleaned once a week.

Fish were fed a diet of large pellet food approximately 50 g/day/tank. The top of the holding tank was covered with a screen to prevent fish from jumping out. A diurnal periodicity of 8 h light/16 h dark was maintained through artificial lighting.

3.3.3 Exposure Aquaria

The exposure set-up consisted of seven glass aquaria (68 L capacity). These aquaria were placed within a cooling bath unit. The cooling water circulated around the bottom 8 cm of each aquarium (Figure 2) maintaining the temperature of the aquarium water between 11-13 °C. The cooling water itself was kept at 6-8 °C by circulation through a refrigeration unit. The aquaria were lined with polyethylene plastic liner bags of 6 mil. thickness. This prevented adsorption of the exposure chemicals onto the silicone joints and glass of the aquarium. Fresh liners were used for each experiment.

Static 8 h exposures were conducted in the aquaria, filled with 50 L of carbon filtered water. The exposure compounds of interest were benzothiophene and dibenzothiophene. Fish ranged from 200 to 350 g in weight and 150 to 300 mm in length.

3.3.4 Exposure Procedure

Prior to an exposure, two fish were placed in each aquarium for a minimum period of 48 h acclimatization (to the aquarium environment). Aeration was provided with diffuser stones and the fish were fed pellets. Feeding was terminated 24 h prior to exposure. Separate aquaria were prepared for the exposures. Fresh water within the

exposure aquaria was aerated with pure oxygen for a period about 10 to 15 minutes (raising D.O. to ≥16 mg/L). Prior to the commencement of the exposure aeration was terminated and the required amount of chemical was then mixed into the aquarium water using a rotary lab.-mixer. The fish (2) were then transferred from their acclimatization aquaria into designated exposure aquaria to begin the exposure. The aquaria were not aerated during the experiment unless the D.O. level fell below 3.7 mg/L (point of visible stress). Water samples were taken at equal intervals from each aquarium during an exposure and stored in teflon sealed vials at 4 °C until analysis could be performed.

3.3.5 Exposure/Depuration Studies:

An exposure concentration of 1.0 mg/L (+/- 0.25 mg/L) was used for each of benzothiophene and dibenzothiophene for a period of 8-9 hrs (refer to Figures 11 to 16). Physical and chemical properties of both benzothiophene and dibenzothiophene are presented in Table 2a. For the purpose of exposure/depuration experiments, fish were removed from the exposure tanks after 8 h and placed in "depuration" aquaria filled with fresh water, for a period of 65 h.

At the end of each experiment, the fish were sacrificed by a blow to the base of the skull. Wet weight and lengths were measured immediately. Each fish was then filletted and all organs individually weighed; muscle, head, viscera, skin and bones and liver. Bile was also extracted (using a 10cc syringe fitted with a 16 gauge, 15 mm needle) from the gall bladder and frozen in micro-centrifuge vials (-80 °C) until required for analysis.

Table 2a. Physical and chemical properties of benzothiophene and dibenzothiophene.

Compound	Melting Point (°C)	Boiling Point (°C)	Solubility in water @ 20 (°C)	Molecular Weight
Benzothiophene	32	221	113 mg/L	134
Dibenzothiophene	99-100	332	17 mg/L	184

3.3.6 Water Analysis

Since benzothiophene and dibenzothiophene had low solubilities in water it was necessary to dissolve them in ethanol and acetone (pesticide grade) respectively. An equal volume of either ethanol or acetone was similarly added to the control tank. Water samples were taken from the aquaria at regular intervals throughout the experiment.

Analysis for dibenzothiophene and benzothiophene in water for trout exposure was performed by high pressure reverse phase liquid chromatography (HPLC) Analysis was performed isocratically using acetonitrile (70%)/ammonium phosphate buffer (pH 6.5, 30%). Volume Injection volume was maintained at 15 μL. Quantitation was performed by the external standard method with benzothiophene and dibenzothiophene stock solutions as the external standard. Area counts obtained for the water samples were compared to the external standard.

3.4 Flesh Samples:

3.4.1 Preparation

Fish muscle was prepared according to the method of Benville and Tindle (1970). Whole frozen fillets were diced into pieces using a knife and mallet. These were then placed in a Waring blender along with 1.5 times as much dry ice. The mixture was then pulverized to a fine powder. The pulverized mixture was then placed in a glass beaker, labelled and sealed with aluminum. It was stored at -80 ^{o}C until required (8 h minimum storage, to allow the dry ice to sublime).

For the soxhlet extraction procedure, 20 g of the thawed powder was vigorously mixed with 80 g of anhydrous sodium sulphate and then packed into a glass thimble (see also Stalling *et al.*, 1972). The thimble contained approximately 2 cm of extracted celite at the bottom. The mixture was extracted with 280 mL methylene chloride for a minimum of 5 h. Following soxhlet extraction, the extract was placed in a refrigerator at ~4 °C, until required. All samples were clearly labelled according to a pre-arranged coding system.

3.4.2 Gel Permeation Columns:

Clean-up of the fish extract followed the method of Hessleberg and Johnson (1972) using gel permeation chromatography (GPC), followed by a florisil partitioning step. Stalling et al. (1972) found GPC to be an effective and reproducible technique for removing lipids from fish extracts. Rainbow trout has a high fat content of about 7-10% of body weight, therefore GPC was considered a suitable technique for removal of the fat. Mulder and Buytynhuys (1970) reviewed applications of gel-permeation to organic compounds and reported separations of a wide range of molecules using bio-beads and various organic solvents; benzothiophene and dibenzothiophene both fall within this molecular range. Both these chemicals, having molecular weights between 130 and 200 are readily separated from lipids, which have molecular weights between 600 and 1500.

The chromatographic column (Figure 3) was prepared by inserting a small amount of glass wool in the bottom of a 750 mm X 19 mm i.d. glass column and packing with bio-beads, SX-3, swollen with elution solvent (Methylene Chloride:Hexane, 1:1). The column was packed by tapping with a glass rod. Tightness of packing and sample texture determined the solvent flow rate during extraction (Hesselberg and Johnson, 1972). The column was topped with 2.5 cm of sea sand for removal of larger lipid particles. A

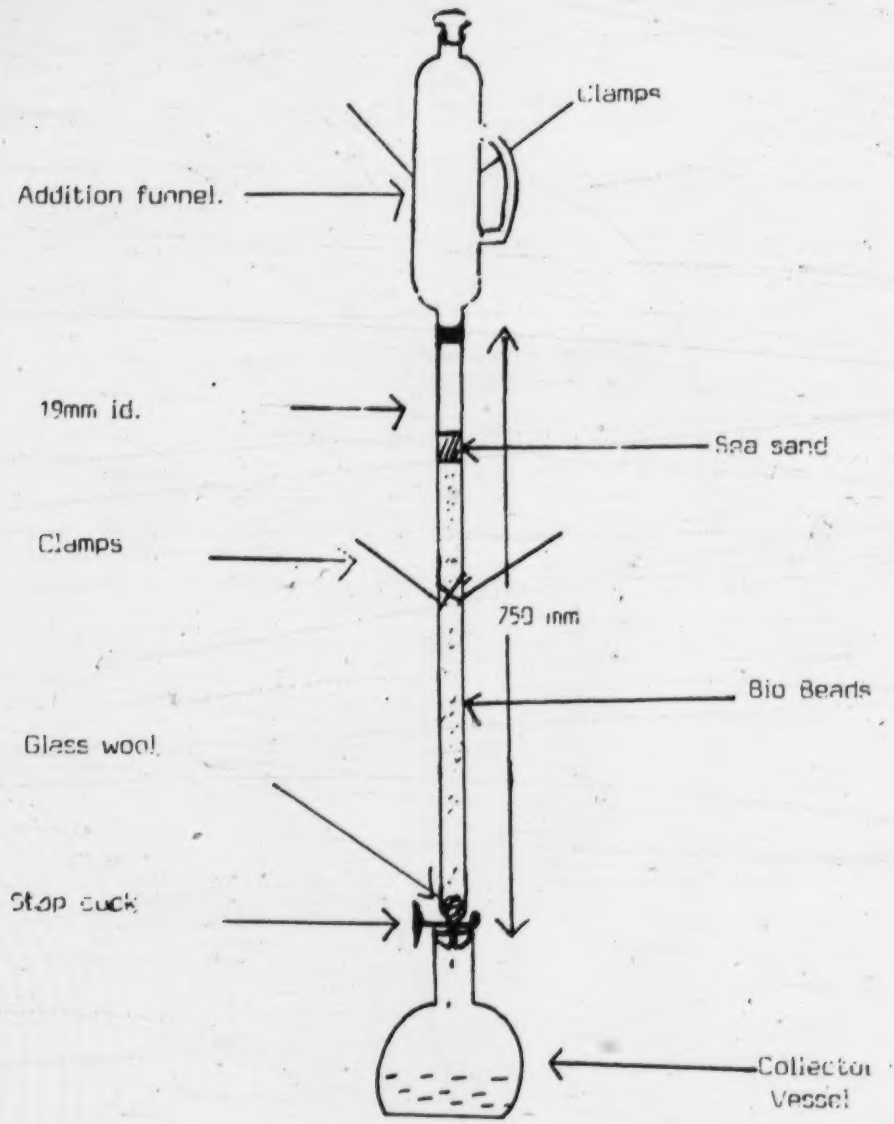


Figure 3. Gel permeation column.

graphical representation of the gel permeation chromatographic column is presented in Figure 3.

3.4.3 Florisil Columns:

Florisil columns (Figure 4) were prepared by activating 10 g (accurately weighed) of 60-80 mesh florisil (Fisher) for 4 h at 650 °C. The florisil was then deactivated by the addition of 5% or 10% deionized water (i.e. 5% water/95% florisil). When not in use, the florisil was stored in scintillation vials and sealed with teflon tape. During an extraction, a small amount of glass wool was placed in the bottom of a 400 mm X 19 mm i.d. glass column and then wet packed with deactivated florisil using hexane as solvent. All glassware was rinsed with hexane prior to use. The florisil was packed with the aid of a glass rod so as to ensure the absence of air. A 2.5 cm layer of sodium sulphate was placed on top of the column so as to remove any water. The hexane level was dropped to within 2 mm of the sodium sulphate layer. The column was then ready for the extract to be cleaned.

3.4.4 Tissue Clean-up:

The tissue extract (280 mL from the soxhlet procedure) was then placed into a 300 mL Kuderna-Danish (K.D.) evaporative concentrator, (refer to Figure 5) and concentrated down to 4 mL. The 300 mL apparatus was then rinsed 3 times with 2 mL of of DCM, for a total of 10 mL of solvent and extract in the Mills tube. This 10 mL was then quantitatively transferred to the head of the GPC column (previously flushed with 100 mL of 50:50 DCM:Hexane) and allowed to run through the column until the extract was within 1-2 mm of the sea sand. The Mills tube was replaced on the K.D. apparatus and the walls rinsed with 5 X 2 mL of DCM. This 10 mL was again run through the GPC column to

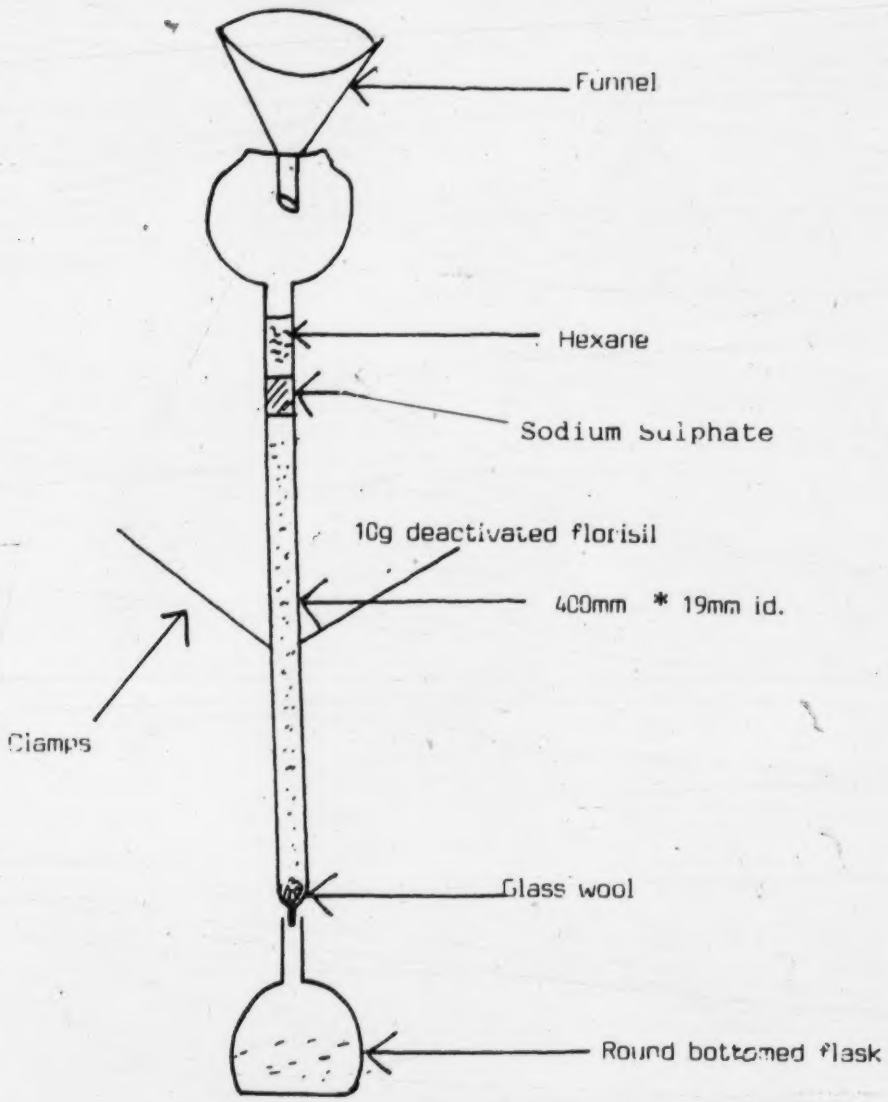


Figure 4. Florisil column.

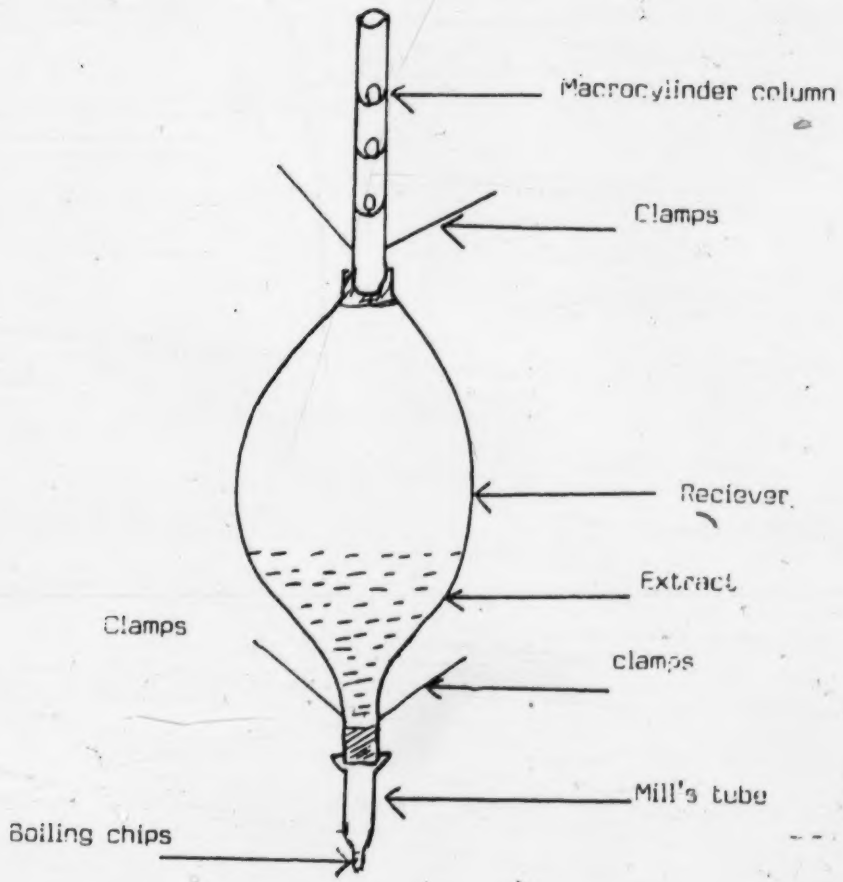


Figure 5. Kuderna-Danish apparatus.

with 1-2 mm of the sand. A 250 mL addition funnel was then placed on top of the column (see Figure 3) and filled with 230 mL of 50:50 DCM:Hexane for elution. The first 80 mL collected was discarded as waste and the next 80 mL retained as sample in a 125 mL round bottom flask.

The 80 mL of eluate retained was then concentrated down to about 4 mL using clean K-D. glassware. The glass wall of the concentrating flask was rinsed with 3X2 mL of DCM, for a total of 10 mL in the Mills Tube. This 10 mL was then quantitatively transferred to the florisil column and eluted to within 2 mm of the sodium sulphate (this step was repeated again with a 3X2 mL rinsing of the mills tube). The florisil column was then eluted with 40 mL of hexane and drained to within 2 mm of the sodium sulphate . All of the extract was collected and transferred to a 125 mL K.D. apparatus.

The eluate was concentrated down to 2 mL and the apparatus rinsed with 3X2 mL hexane. This 8 mL was then transferred to a 15 mL centrifuge tube, in addition to 2X2 mL of hexane rinse from the Mills tube. This final extract was concentrated to 1 mL with the aid of a Model III Nitrogen Evaporator.

The tissue extract was analysed for benzothiophene and dibenzothiophene by GC/FID and GC/FPD using a HP model 5880 and Varian model 3500 GC respectively.

3.5 Liver Sample Preparation:

All liver samples were analyzed for benzothiophene and dibenzothiophene using a modification of the method of Varanasi *et al.* (1982a). Potassium phosphate buffer (pH 7.0) was used instead of saline. Frozen liver (2-4 g) was thawed and transferred to a 50 mL centrifuge tube with 4 mL of potassium phosphate buffer (pH 7.0). The mixture was

homogenized with a polytron (Brinkman instruments) for approximately 1 min.. The polytron probe was then washed with 5X2 mL methanol into the centrifuge tube. Chloroform (5 mL) was then added and the mixture vortexed for 1 min. This step was repeated followed by the addition of 5 mL of organic-free water. The mixture was vortexed for 1 min. and then centrifuged at 2000 rpm for 5 min. (Centrifuge Model C.L. (I.C.S.). After centrifuging, there was three distinct layers: an upper water layer, middle solids layer and a lower chloroform/methanol layer. The lower chloroform/methanol layer was removed and dried through a 10 g sodium sulphate column and collected in a 50 mL centrifuge tube. The aqueous/solids layer was then extracted with 10 mL chloroform and vortexed for 1 min. The lower chloroform layer was removed and dried through the sodium sulphate column. The extract was then concentrated to 1 mL on the N-evaporator as the final step in preparation for analysis by GC. The aqueous portion was saved for further analysis.

3.5.1 Liver Metabolites

The aqueous-methanol phase of the liver extraction (with protein removed by centrifugation) was evaporated to 6 mL with the nitrogen evaporator and made up to 0.2M in acetate, buffered at pH 5.0. It was incubated with 2000 units of b-glucuronidase (containing sulphatase activity) for 4h at 37°C following the procedure of Varanasi *et al.* (1982a).

Following hydrolysis, the solution was extracted with 10 mL methanol and vortexed for 1 min. This was followed by 2X5 mL chloroform with vortexing and 5 mL organic free water. The lower chloroform-methanol layer was dried through a 10 g sodium sulphate column and collected in a clean 50 mL centrifuge tube. A further extraction with 10 mL chloroform was performed, the lower layer dried through the sodium sulphate

column and the resulting 35 mL of extract concentrated to 1.0 mL with the aid of a N-evap. It was then analysed on a Varian model 3500 GC, with FPD detector.

3.6 Bile Analysis:

3.6.1 Enzymatic hydrolysis of bile

Bile samples were analysed for conjugated metabolites by the following method: an aliquot of bile (100 mL) was placed into a 15 mL centrifuge tube and dissolved in 0.5 mL of a 0.2M acetate buffer. This mixture was incubated together with 2000 units of b-glucuronidase (dissolved in 0.1 mL of the acetate buffer) for 2 h at 37°C, in a shaking incubator.

After enzyme treatment, the solution was extracted with chloroform-methanol according to the following procedure:

-2.5 mL of methanol and 1.25 mL of chloroform were added to the solution and vortexed for 1 minute.

-an additional 1.25 mL of chlorpform was added to the vessel and vortexed for 1 min., followed by 1.25 mL of organic free water with vortexing.

-two layers are formed; the bottom phase being chloroform. This lower layer was removed and dried by passage through a 1 g sodium sulphate column and collected in a 5 mL calibrated centrifuge tube.

-the remaining aqueous phase (upper layer) was further extracted with an additional 2 mL of chloroform, vortexed and the lower layer dried through the sodium sulphate column as before.

-the combined extracts were concentrated down to 1.0 mL on the Nitrogen-evaporator and the extract analysed on the Varian model 3500 GC, with FPD.

-parent compounds found in extracts were quantified using acenapthene as an internal standard.

3.6.2 Acetylation of bile

Enzymatically hydrolyzed and extracted bile samples were acetylated according to the procedure of Hargesheimer *et. al* (1981). The bile (100 mL) was placed in a 25 mL centrifuge tube, along with 4 mL demineralized water and vortex for 1 min. Solid sodium bicarbonate was slowly added until there was an excess of sodium bicarbonate and the evolution of carbon dioxide ceased. This solution was then extracted with 3X2 mL of chloroform and the chloroform layer dried through a sodium sulphate column. The resulting extract was concentrated to 1 mL on the nitrogen evaporator. The sample was analysed on the HP model 5880 GC/FID and the Varian model 3500 GC/FPD.

3.6.3 Caustic hydrolysis of bile

Caustic hydrolysis of bile was performed in the search for first pass metabolites. Aliquots of bile (100 mL) were placed in a 15 mL centrifuge tube along with 1 mL of 6N sodium hydroxide. This mixture was vortexed for 1 min. and then extracted with chloroform and methanol according to the procedure outlined in pp. . The final extract was analyzed using GC/FPD.

3.7 Tainting Potential of Benzothiophene:

3.7.1 Apparatus

All test were performed in an odour-free room located in the Department of Food Science , in the University of Alberta. This sensory testing laboratory was equipped with individual booths and red lights to disguise possible color differences between samples as outlined in ASTM (1986). A constant temperature of 21°C and a relative humidity of approximately 50% was maintained. The panel booth design followed the guidelines as set out in ASTM (1986) as did the plan of the sensory testing laboratory. Panelists were given tap-water and dilute lemon solution as rinsing material and unsalted soda crackers (as outlined by Iredale and York, 1976) were provided to allow the judges to clear the previous sample from their palates before continuing . Glassware was cleaned with hot soapy water and rinsed several times with fresh water. Samples were kept warm in a glass 2L beaker filled with water placed on top of a hot plate. Napkins and miniature garbage bags were supplied to each panelist. Equipment used in testing was stored in the odour-free room and used only for these tests as outlined by Alexander *et al.*, 1982. All samples were prepared in a sample preparation area connected to the sensory lab by a cupboard door pass-through.

3.7.2 Ethics:

An ethics committee was set up in the University of Alberta to review the research projects of three graduate students (including this project) using humans as sensory "guinea pigs". Prior to any sensory analysis, statements on the conduct of the tests were examined and approved by this committee. The statements assured that:

- the individual panelists were thoroughly instructed on techniques they were to employ.
- the panelists were supervised to preclude consumption of the fish. Any deviations from this policy by panelists were to be documented in writing.
- there were procedures to abort the test if a panelist complained of any discomfort.
- there were procedures to ensure prompt medical action should the latter occur.

3.7.3 Panelists:

Judges chosen for the study were picked from an original group of 27 people on their ability to be consistent organoleptically and to identify an "off-flavor" in fish. The panelists ranged in ages from 20 to 39 and the majority were graduate and undergraduate students at the University of Alberta. Each selected judge signed a waiver and release form prior to the sensory analysis of experimental fish. A copy of this release and waiver is supplied in Appendix 1. In addition, all panelists completed a personal medical information document, to ensure the researchers that no medical complications would interfere with the sensory analysis. A copy of this form is supplied in Figure 6. Each panelist was thoroughly instructed on the techniques employed and was supervised during the actual procedure.

3.7.4 Screening and training tests

All panelists were carefully screened and trained prior to the actual sensory evaluations as outlined by Jellinek, 1985. The work involved in screening and training the

PERSONAL HISTORY

Name: _____

Address: _____

_____

Telephone: _____

Age: _____ Sex: _____

Do you have any history of cardiac or respiratory ailments? (Yes or No)

_____

If yes, please provide details below:

_____

_____

_____

Do you have any allergies? (Yes or No) _____

If yes, please provide details below:

_____

_____

_____

Were you ever a habitual smoker of cigarettes? (Yes or No) _____

If yes, when, approximately, did you quit? _____

Figure 6. Personal medical history form.

panelists was shared by two other graduate students. The screening process consisted of two phases: the first phase measured both the ability of each panelist to distinguish the four basic tastes of sweet, sour, bitter and salty (Jellinek, 1985 and Piggott, 1984) (Table 3); and to distinguish and describe familiar odours (Table 4) as outlined by Amerine, *et al.* (1963). The second phase consisted of a series of tests in which two actual fish samples were compared and the panelist was asked to determine which sample was more "tainted". This was a forced-choice procedure, disallowing a "no-difference" response. The tainted sample was fortified with naphthalene. In the second phase of panel sessions, each judge tasted seven pairs of samples (Figure 7), each pair evaluated consisted of one sample of the "untainted" fish and one of the "tainted" fish. The samples were all coded according to the randomized design . Questionaires for the screening tests are supplied in Appendix 2. The 11 selected panelists (out of an original group of 27) were chosen because of their ability to consistently discriminate the different tastes and odours, as well as their high scoring of correct responses. The selected panelists were then required to go through training sessions to familiarize them with the particular testing procedure and the medium being investigated.

3.7.5 Questionaire for benzothiophene:

The procedure used was a modification of the quantitative descriptive analysis of Stone *et al.* (1974) and Lockhart *et al.* (1986). Each panelist was presented with eight pairs of coded samples. Panelists were asked to record their response to each sample by placing a check mark inside a "rating box" which corresponded to the intensity of the off-flavor in the sample. (refer to sample questionaire, Figure 8). These responses were later converted to numerical scores using a template. Each sample was evaluated 3 times by each panelist. Mean scores of fortified and exposed fish are given in Table 14 in section 4.6.2.

Table 3. List of suggested compounds for taste standards.

Sweet	*Sour*	*Salt*	*Bitter*
glucose	acetic acid	NaCl	Caffeine
Sucrose	Citric acid	NH_4Cl	Nicotine
Almonds	Malic acid	$MgCl_2$	Quinine
Lactose			

Table 4. List of suggested standards for odors. The list has been compiled from Jellinek, 1985 and Piggott, 1984.

Odor	*Standard*
Bitter	Quinine
Earthy	Geosmin
Chlorinous	Na-Hypochlorite, Bleach
Onion	Isopropylmercaptan
Medicinal	Iodine
Model paint	Methyl-isobutylketone
Cloves/spicy	Cloves
Soapy	Soap
Grassy/rotten nuts	Hexanal
Moth balls	Naphthalene
Sweet	Vanilla
Sweet	Almonds
Sweet	Nonal
Model glue	Toluene
Fishy	Tuna
Oily	Oil sands effluent

SCREENING TEST FOR FISH TAINTING

NAME_____ DATE_____

Seven pairs of coded samples are provided. Each of these foil packets contains a sample of fish. Some samples have been artificially "tainted" with varying concentrations of a common household substance (moth balls). Other samples are untainted.

To evaluate the samples, tear off the end of the foil packet and open it as much as possible. Sniff each pair of samples separately and in the order indicated. Circle the sample within each pair which is most tainted. After you have completed your evaluation, close each packet by folding over the open end.

Rinse your mouth with the lemon water provided and take a bite of cracker after smelling each sample pair. Wait approximately 15 seconds between sample pairs.

Pair	Samples	
1	321	332
2	621	448
3	554	416
4	481	197
5	871	763
6	223	323
7	787	576

Figure 7. Questionaire for phase 2 of screening process.

FISH FLESH SENSORY EVALUATION RUN #2

NAME _____ DATE _____

Eight pairs of cooked fish samples are provided. In each pair, one of the samples contains fish that has been exposed to benzothiophene only. The other sample contains fish that has either been exposed to dechlorinated tap water or spiked with benzothiophene. In order to evaluate the samples, tear off the end of the aluminum packet and open it as much as possible. Sniff each pair of samples individually and in the order indicated. If a taste cannot be detected by sniffing alone, you are requested to taste the sample by placing it in your mouth for approximately 20 seconds. Please expectorate it after this time. You are requested not to swallow the sample. Circle the sample within each pair which is most tainted. Evaluate the degree of taint of this sample by ticking within the most appropriate category (see example below). Rinse your mouth with the lemon water provided, and take a bite of cracker after evaluating each pair of samples. Wait approximately 15 seconds between pairs.

Pair No.	Samples		Degree of Taint
Example	770	(888)	barely detectable — detectable — highly detectable
1	832	810	barely detectable — detectable — highly detectable
2	608	671	barely detectable — detectable — highly detectable
3	444	498	barely detectable — detectable — highly detectable
4	855	826	barely detectable — detectable — highly detectable
5	233	307	barely detectable — detectable — highly detectable
6	901	806	barely detectable — detectable — highly detectable
7	308	777	barely detectable — detectable — highly detectable
8	221	225	barely detectable — detectable — highly detectable

Figure 8. Questionaire for benzothiophene.

3.7.6 Preparation of fish: fortified samples

Frozen flesh was thawed in cold running water (while still in vacuum sealed bags) and ground in a Waring blender. The minced flesh was fortified with the required amount of chemical dissolved in ethanol (95% ethanol, 5% water; Fisher grade). The desired amount was calculated on a mg/kg basis and stock solutions were prepared. For example, from a 1000 ppm stock solution of benzothiophene in ethanol, 5X200 µL aliquots were added to 1000 g of fish flesh to obtain a 1 ppm sample. Each 200 µL was added separately and then blended in the stainless steel Waring blender to obtain as homogeneous a sample as possible. An equal amount of ethanol was added to the control samples as was used above for dissolving the benzothiophene.

3.7.7 Preparation of flesh: exposed samples

In the case of exposed flesh, fish were exposed to varying concentrations in aquaria (Section 3.3.4). The concentrations used were 0.01, 0.03, 0.08, 0.5 and 1 ppm. The fish were exposed under static conditions in a non-aerated tank for 8 h. They were then killed by a blow to the base of the skull, eviscerated, washed in distilled water and the fillets vacuum sealed and frozen at -20 °C until required. Water samples were not taken during these experiments as we were only interested in flesh concentration. Analysis of the flesh by GC/FPD indicated that these samples needed to be diluted with control fish flesh to give an actual concentration of 0.03, 0.08, 0.5 and 1.0 ppm. Exposed flesh was diluted with control flesh by mixing both together in the Waring blender, until the required concentration was obtained (as determined by GC/FID analyses of the samples). This procedure was necessary for all but the 0.01 ppm concentration, as samples of exposed fish were available which contained a level of 0.01 ppm. Finally, an equal amount of ethanol was added to the exposed fish as to the fortified fish, in order to nullify any

discrepancies caused by the ethanol. All prepared flesh was re-analyzed by G.C./F.I.D. prior to sensory evaluation to ensure the correct concentration.

3.7.8 Sample Preparation

Individual samples for sensory analysis were prepared according to the procedure of Iredale and York (1976) and Lockhart, *et al.* (1986). For each sample, a 15 g portion was wrapped in aluminum foil and the aluminum coded with a wax pencil for identification during sensory analysis. Each pair of samples was individually vacuum sealed, labelled and frozen. Eight pairs were placed in one larger bag and frozen until required.

For presentation, the wrapped, coded samples (8 pairs) were heated in water at 60 ^{o}C for 10 minutes and served in a beaker of water on a warming plate. The panelists were instructed to open each sachet containing one pair and to compare the two samples. A copy of the questionaire is provided in Figure 8.

4. Results

4.1 Quality Control:

Portions of tissue (muscle and liver) were obtained from control fish and analyzed before and after fortification with benzothiophene and dibenzothiophene. Recovery of benzothiophene and dibenzothiophene from fortified tissue (liver and muscle) is summarized in Tables 5 and 6. From these results it is apparent that acceptable levels of both benzothiophene and dibenzothiophene were obtained from fortified fish tissue. The chromatograms of the liver samples indicated a large co-elution of biogenic material (Figure 9); however, the level of this interference was not so high as to warrant further sample cleanup.

The efficiency of the enzymatic hydrolysis was tested by hydrolyzing replicate aqueous solution of α-Napthyl-β-D-glucuronic acid (200 βg/mL) and extracting and analyzing the resulting solution for 1-napthol by GC/FID. The hydrolysis was found to be greater than 95% effective. The efficiency of the GPC columns was tested by eluting the columns with 1 mL of standard solutions of benzothiophene and dibenzothiophene (100 μg/mL) and analyzing the eluates for percent recovery. Figure 10 depicts the chromatograms obtained from the standard as compared to the quality control. Recovery efficiency was found to be 98%.

Analysis of tissue (muscle and liver) as well as bile, was performed on both GC/FPD and GC/FID. The former was used primarily to identify sulphur compounds whereas the latter was used to quantify amounts because, the linear response of the GC/FID, being independent of the shape of the peak, is more favorable to use in quantifying amounts. The FPD when used in the sulphur mode is inherently non-linear

Table 5: Recovery of benzothiophene from fortified fish tissue for the purpose of quality control.

Tissue	*Replicate Analysis*	*Fortification Level (µg/g)*	*Mean Recovery benzo (%)*
Muscle	3	1.0	97
Liver	2	4.5	90

Table 6: Recovery of dibenzothiophene from fortified fish tissue for the purpose of quality control.

Tissue	*Replicate Analysis*	*Fortification Level (µg/g)*	*Mean Recovery benzo (%)*
Muscle	3	1.0	97
Liver	2	4.0	93

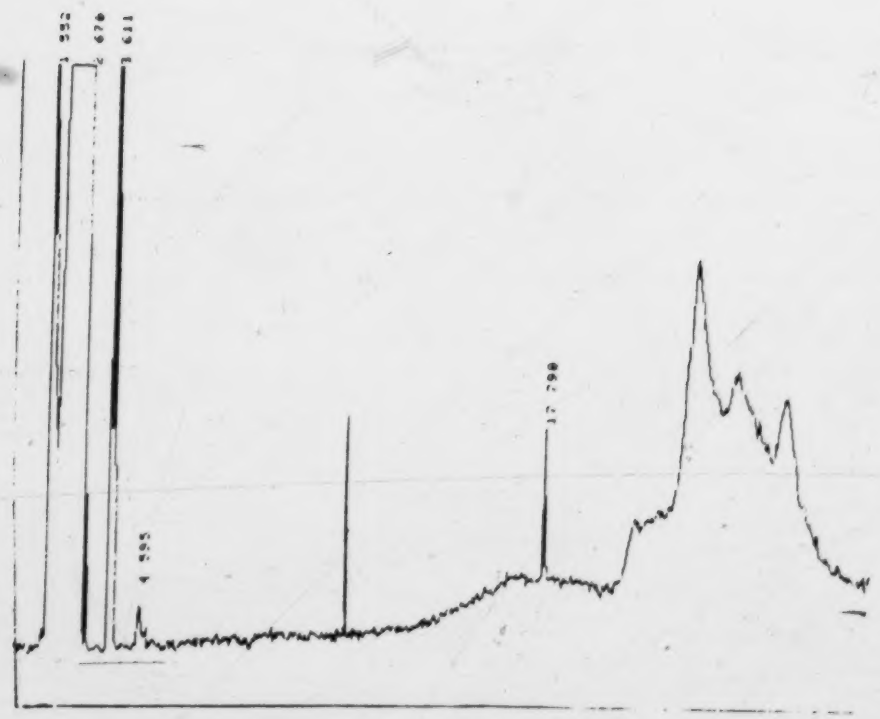


Figure 9. Chromatogram of fortified liver.

Figure 10. Gc/fpd chromatograms of (a) quality control eluate from GPC and (b) standard of comparison

(Attar *et al.*, 1977; Bowmann and Benoza, 1968) as a result both the maximum height and the area will be a function of the shape of the peak resulting in large errors.

4.2 Benzothiophene and Dibenzothiophene in Water:

The average concentrations of benzothiophene and dibenzothiophene in water, as determined by HPLC, were 0.7 and 0.52 mg/L respectively. Water samples were taken at regular intervals during the exposures. There was a significant drop in concentration from the start to the finish of the experiment in both cases (by as much as 50% in the case of some tanks, see Figures 11 to 16). This drop in concentration is results from the volatility and low solubility of both compounds. Water solubilities for benzothiophene and dibenzothiophene are 113 and 1.7 mg/L respectively. Additional losses of the chemical may have occurred through adsorption of the chemicals onto the polyethylene liners of the aquaria. The most dramatic decrease occurs in the first three hours; thereafter the concentration levels off somewhat.

4.3 Benzothiophene:

4.3.1 Accumulation and elimination studies

Table 7 illustrates the tissue distribution of fish exposed to benzothiophene. Table 8 illustrates the tissue distribution of exposed fish followed by 65 h depuration. Fish exposed to benzothiophene in water readily accumulated the chemical, but they can depurate this substance to a large extent after 65 h in fresh water. Mean bioconcentration factors were determined to be 37, 45 and 29 for the muscle, liver and bile respectively. These were calculated by dividing the mean tissue concentration by the mean exposure concentration.

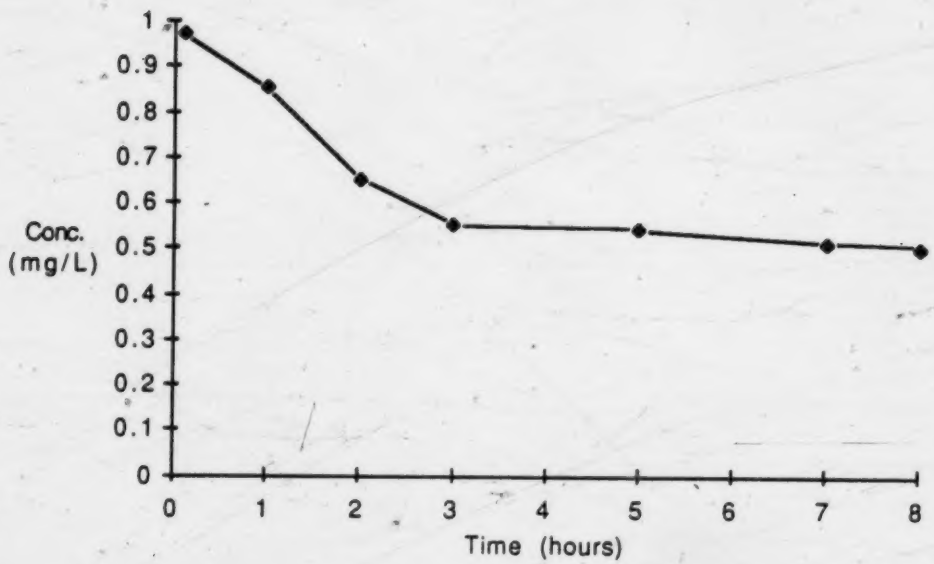


Figure 11. Benzothiophene exposure concentration in Tank #1, with two fish present.

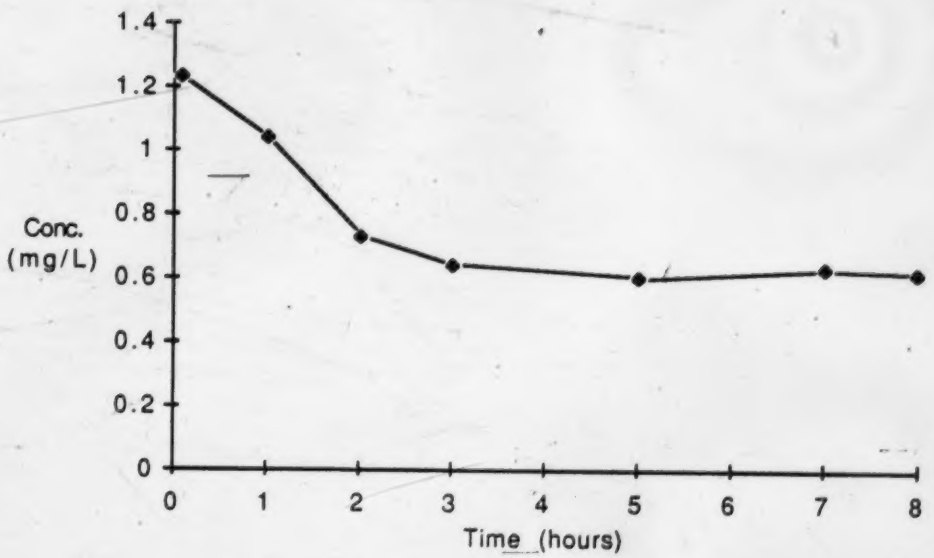


Figure 12. Benzothiophene exposure concentration in Tank #2, with two fish present.

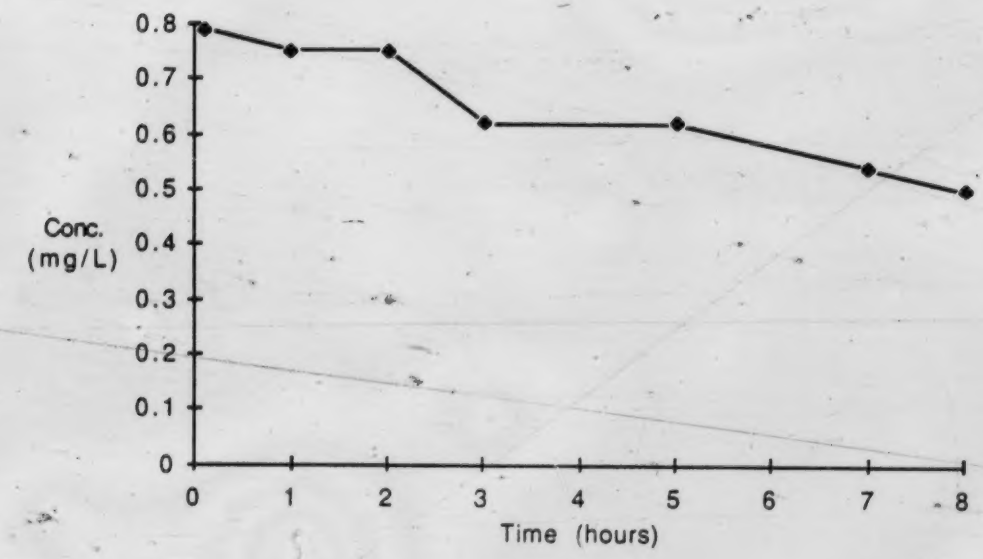


Figure 13. Benzothiophene exposure concentration in Tank #3, with two fish present.

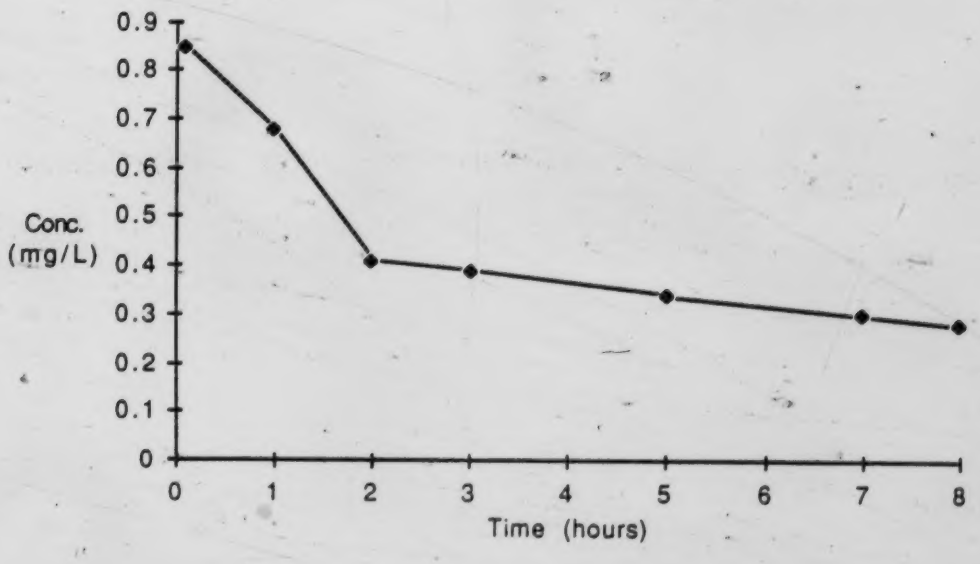


Figure 14. Dibenzothiophene exposure concentration in Tank #1, with two fish present..

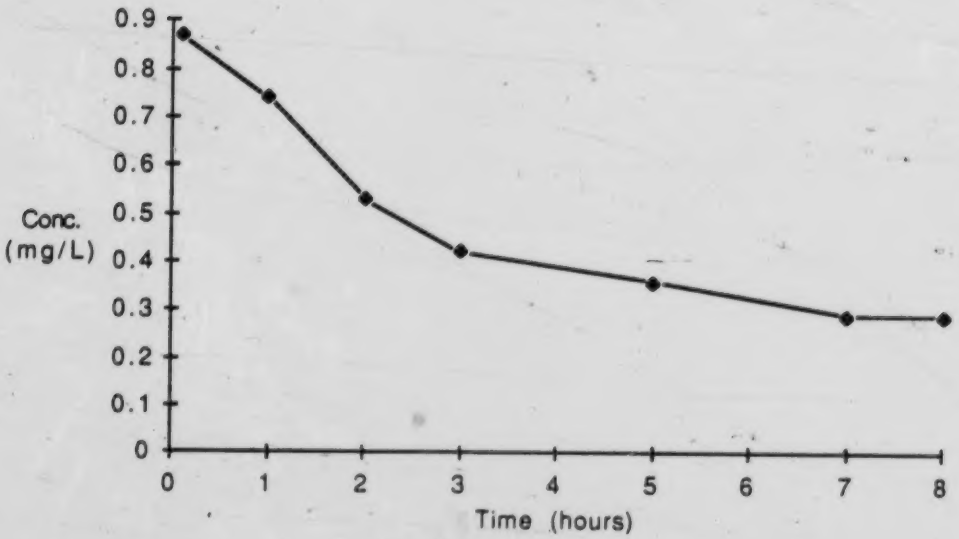


Figure 15. Dibenzothiophene exposure concentration in Tank #2, with two fish present.

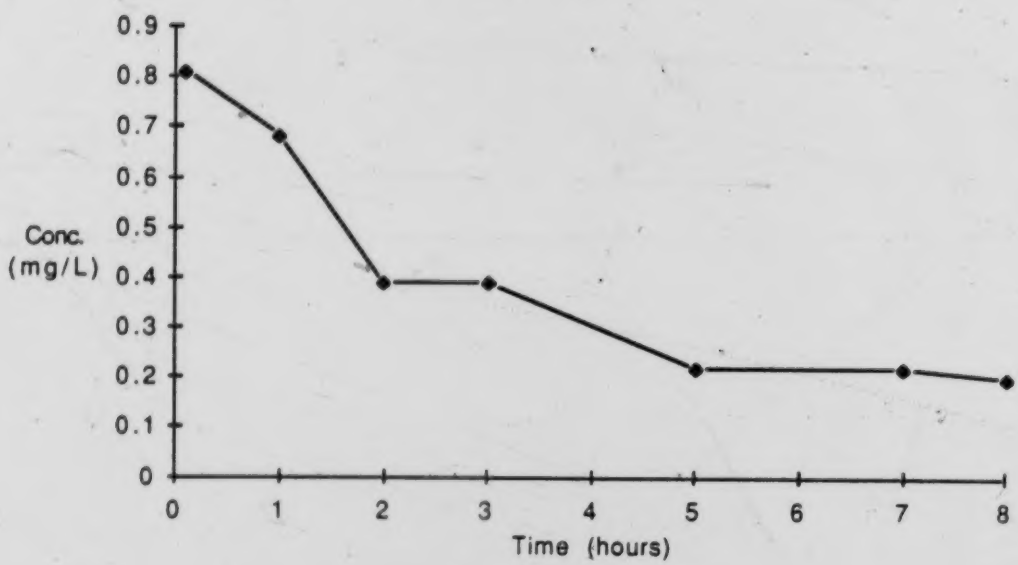


Figure 16. Dibenzothiophene exposure concentration in Tank #3, with two fish present.

Table 7. Distribution of benzothiophene in exposed fish (no depuration).

Replicate #	1	2	3
Exposure time (h)	8	8	8
Mean Exposure concentration (mg/L)	0.67	0.81	0.55
Conc. Range (mg/L)	0.99-0.50	1.23-0.64	0.78-0.54
Length of fish (mm)	272	242	271
Weight of fish (g)	254	163	261
Muscle weight (g)	139	89	133
Muscle conc. (µg/g)	18.7	30.6	26.3
Liver weight (g)	2.9	2.4	4.0
Liver conc. (µg/g)*	24.3	36.2	31.0
Bile conc. (µg/g)**	5.4+	10.3	3.9

* The quantities of benzothiophene found in the liver were from direct chloroform methanol extraction. Treatment of the liver sample with B-glucuronidase indicated an absence of the parent compound.

** The represents the concentration found in the bile after enzymatic hydrolysis followed by chloroform methanol extraction.

+ This particular extract evaporated to dryness in the vial before analysis: thus the residue was diluted with 50 mL of chloroform prior to analysis. This value, therefore represents a conservative estimate of the actual concentration.

Table 8. Distribution of benzothiophene in fish with 65h depuration.

Replicate #	1	2	3
Exposure time (h)	8	8	8
Mean Exposure concentration (mg/L)	0.67	0.81	0.55
Conc. Range (mg/L)	0.99-0.50	1.23-0.64	0.78-0.54
Length of fish (mm)	256	232	290
Weight of fish (g)	217	171	300
Muscle weight (g)	103	89	121
Muscle conc. (μg/g)	2.25	1.6	4.9
Liver weight (g)	3.0	2.6	4.5
Liver conc. (μg/g)*	2.5	1.7	1.3
Bile conc. (μg/g)**	0	0	0

* This value represents that concentration found in the bile after enzymatic hydrolysis followed by chloroform methanol extraction.

** This value represents that concentration found in the liver after direct extraction (no enzyme treatment).

Evidently the liver bioconcentrates benzothiophene to the greatest extent. One liver chromatogram obtained from a fish exposed to the hydrocarbon, is presented in Figure 17. The concentration in the liver dropped from 31.1 µg/g to 1.8 µg/g following depuration and that of the muscle dropped from an average of 25.2 µg/g to 2.8 µg/g, There was no evidence of the parent compound in the bile after depuration, whereas levels immediately after exposure averaged 6.5 µg/g. Chromatograms of bile and flesh from fish exposed to benzothiophene (in addition to controls) are presented in Figures 18 and 19. The parent compound is eluted from the column at 10.11 mins. Parent compound observed in livers and bile of these fish, were positively identified using GC/FID in conjunction with GC/MS. The total ion chromatogram of one fish sample is given in Figure 20. The spectrum appearing at 8.18 min. was identified as the parent compound, because the observed spectrum and retention time matched that observed for reference material. The retention time on the GC/MS differs from that of the GC/FID because the columns in both are dissimilar; temperature programming is also different in both these GCs.

Despite depuration, detectable levels of the chemical were present in the muscle and liver. Mean concentrations in both were determined to be 2.9 and 1.8 µg/g respectively. From these results, it would appear that benzothiophene is eliminated to a large extent (89-100%) from muscle, liver and bile.

4.3.2 Metabolism studies : benzothiophene:

Liver and bile from fish exposed to benzothiophene were analyzed for evidence of metabolism. Both tissues were enzymatically hydrolyzed using glucuronidase solution containing sulphatase activity. Following hydrolysis, a chloroform-methanol extraction was performed (refer to methods section). However, analysis of the extracts revealed parent compound only with little evidence of conjugated metabolites. Direct acetylation and

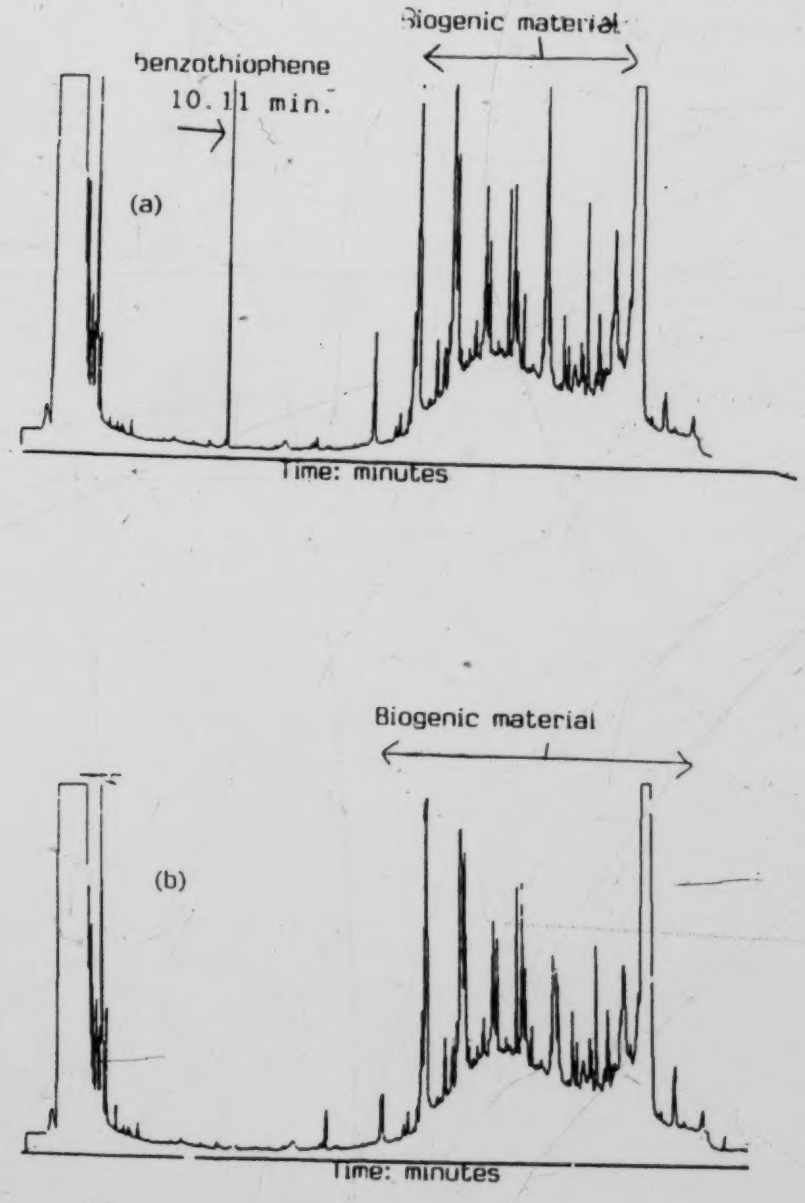


Figure 17. GC/FPD chromatograms from liver of fish (a) exposed to benzothiophene (b) control fish.

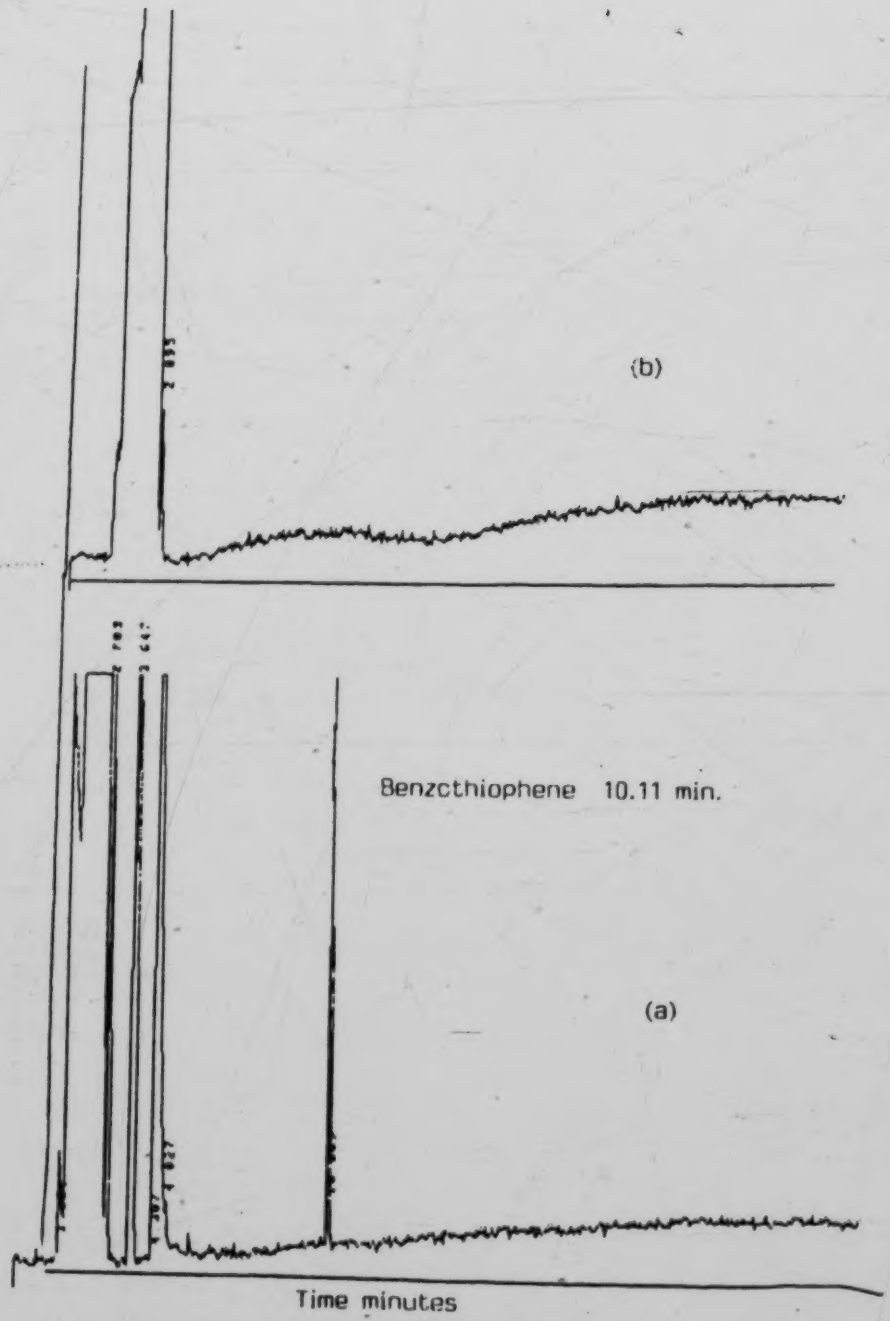


Figure 18. Chromatogram of bile from; (a) fish exposed to benzothiophene, and (b) control fish.

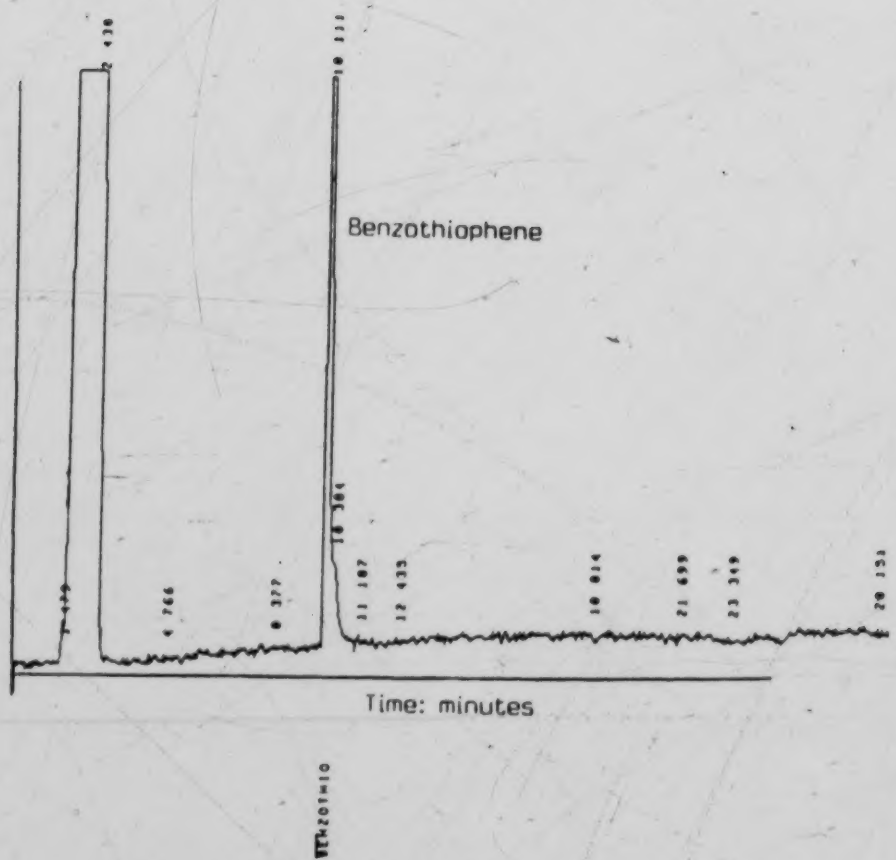


Figure 19. Chromatogram of flesh from fish exposed to benzothiophene

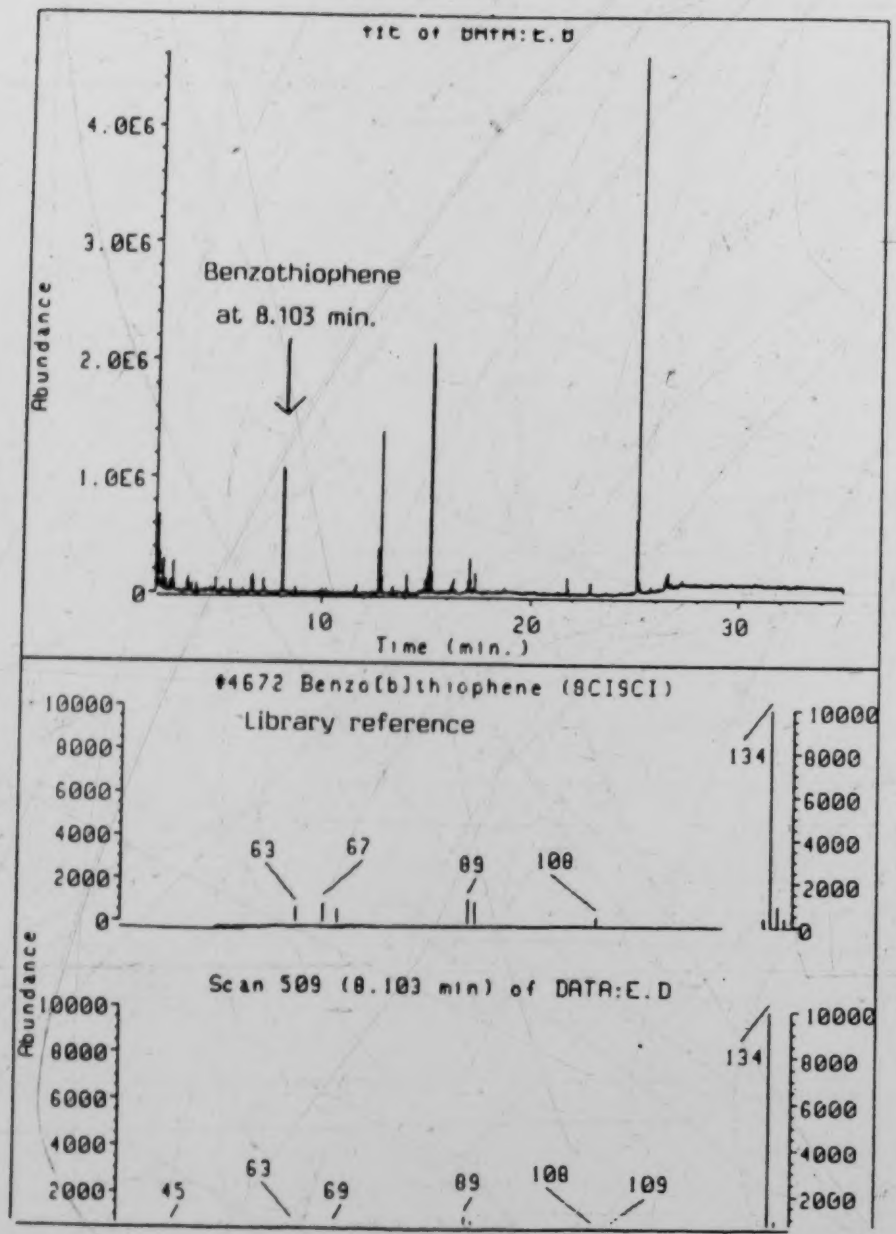


Figure 20. Total ion chromatogram of flesh exposed to benzothiophene Scan of peak at 8.103 min. matches this compound with that of reference benzothiophene.

caustic hydrolysis was also performed on bile samples in search of metabolites, but none were found. One possible pathway for the mobilization of benzothiophene is represented in Figure 21. However, no glucuronide conjugates could be detected by analysis.

4.4 Dibenzothiophene

4.4.1 Accumulation and elimination studies.

Table 9 illustrates the tissue distribution observed for fish exposed to dibenzothiophene. Table 10 illustrates the tissue distribution of exposed fish followed by 65 h depuration. Table 11 shows that rainbow trout can readily accumulate dibenzothiophene in muscle and liver. Mean bioconcentration factors were 43 and 32 for muscle and liver respectively. Mean concentrations in the muscle and liver were 23 and 18.5 mg/g respectively. Chromatograms of liver and flesh from fish exposed to dibenzothiophene are presented in Figures 22 and 23. They are compared to control samples. As can be seen from these figures, the parent compound is eluted from the column at 17.798 mins. The parent hydrocarbon was positively identified using G.C./MS in conjunction with GC/FID. Standards of benzothiophene were used to positively identify the chemical in GC/FID, whereas a comparison with library references was used on the mass spectrometer. An example of one total ion chromatogram is presented in Figure 24. The spectrum appearing at 15.403 min. was correlated with the parent compound, as it matched library reference material.

Both an enzymatic hydrolysis and a caustic hydrolysis were performed on the bile in search of parent compound. There was no evidence of the parent compound in the bile. This would lead one to suspect that the chemical had possibly been metabolized in the liver prior to deposition in the bile.

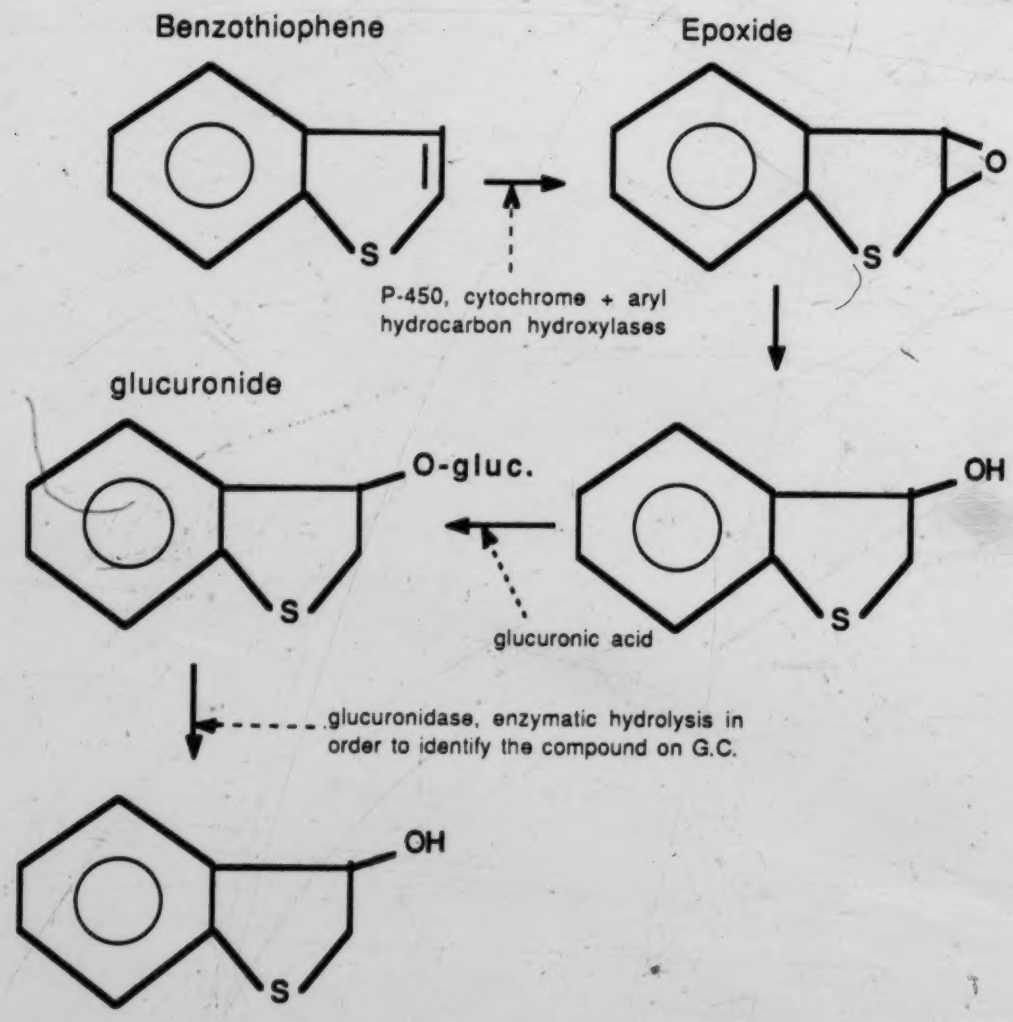


Figure 21. Graphical representation of suspected metabolic pathway of benzothiophene. In this scheme benzothiophene is converted to the epoxide via the cytochrome P450 enzyme system and converted to the monohydric alcohol followed by an additional reaction of the molecule: glucuronic acid is incorporated into the molecule. This compound is then enzymatically hydrolysed in the laboratory with glucuronidase in order to identify the alcohol by GC-FID. However, no standard was available in the laboratory for comparison and this compound was not identified.

Table 9. Distribution of dibenzothiophene in exposed fish (no depuration).

Replicate #	1	2	3
Exposure time (h)	8	8	8
Mean Exposure concentration (mg/L)	0.53	0.56	0.49
Conc. Range (mg/L)	0.86-0.42	0.86-0..27	0.81-0.49
Length of fish (mm)	236	195	227
Weight of fish (g)	162	102	143
Muscle weight (g)	81	45	73
Muscle conc. (μg/g)	26.6	24.0	18.2
Liver weight (g)	3.1	2.5	1.6
Liver conc. (μg/g)*	13.8	18.9	23.0
Bile conc. (μg/g)**	0	0	0

* This concentration represent that found in the liver after direct extraction (no enzyme treatment).

** This concentration represents that found in the bile after enzymatic hydrolysis followed by chloroform methanol extraction.

Table 10. Distribution of dibenzothiophene in exposed fish with 65h depuration.

Replicate #	1	2	3
Exposure time (h)	8	8	8
Mean Exposure concentration (mg/L)	0.53	0.56	0.49
Conc. Range (mg/L)	0.86-0.42	0.86-0..27	0.81-0.49
Length of fish (mm)	257	254	278
Weight of fish (g)	232	201	256
Muscle weight (g)	72	144	37
Muscle conc. (μg/g)	10.5	11.2	8.8
Liver weight (g)	5.0	5.4	1.3
Liver conc. (μg/g)*	16.8	12.3	13.7
Bile conc. (μg/g)**	0	0	0

* This concentration represent that found in the liver after direct extraction (no enzyme treatment). Enzymatic hydrolysis was performed on the liver sample, but indicated an absence of parent compound.

** This concentration represents that found in the bile after enzymatic hydrolysis followed by chloroform methanol extraction.

Table 11. Bioconcentration factors for benzothiophene and dibenzothiophene in muscle, liver and bile of exposed fish. These were calculated by dividing the mean water concentration by the mean tissue and bile concentrations.

Compound		Bio-Concentration Factors		
		Muscle	*Liver*	*Bile*
Benzothiophene	1	28	36	8
	2	38	45	13
	3	48	56	7
Dibenzothiophene	1	50	26	-
	2	43	34	-
	3	37	47	-

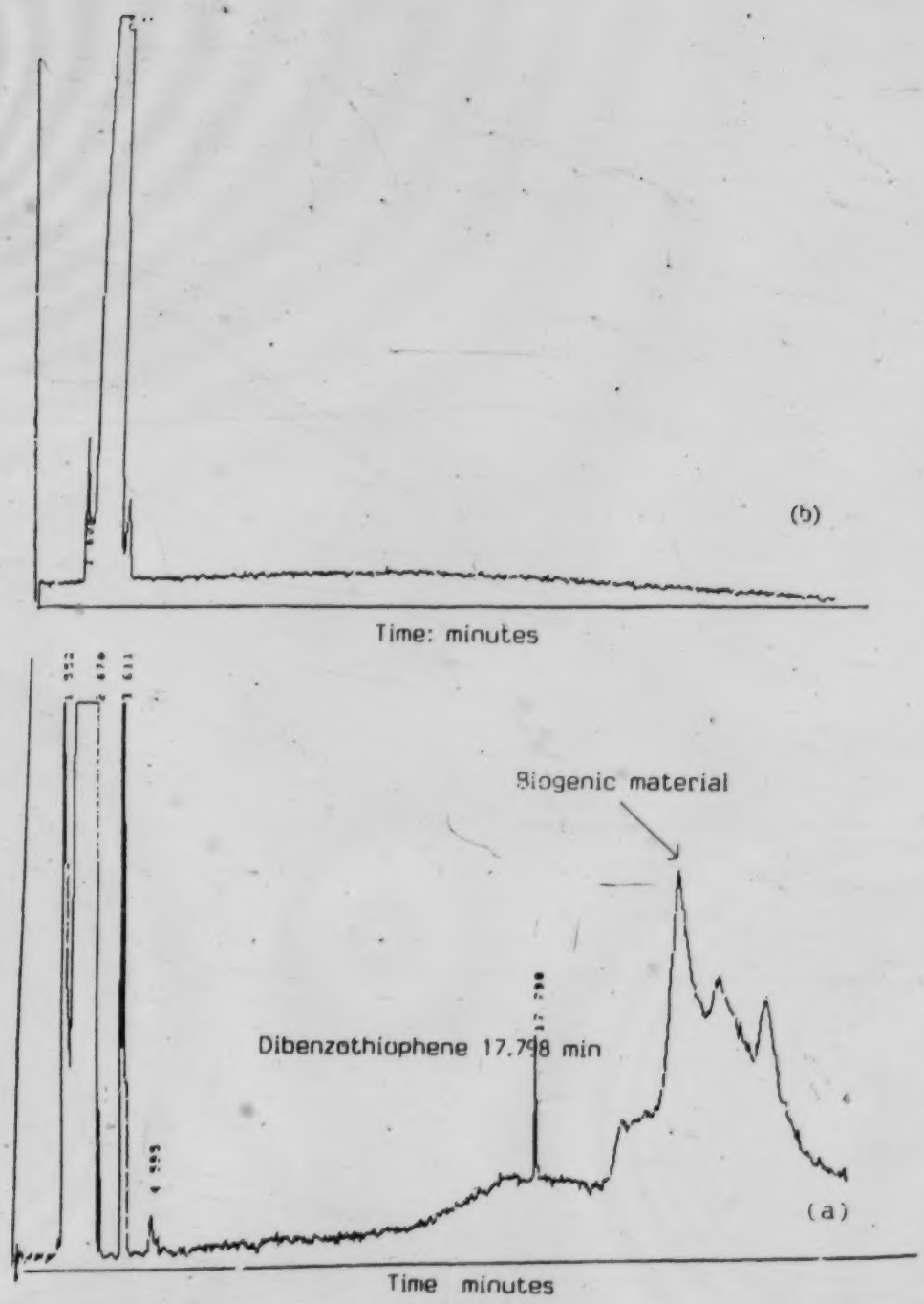


Figure 22. Chromatogram of liver from; (a) fish exposed to dibenzothiophene, and (b) control fish.

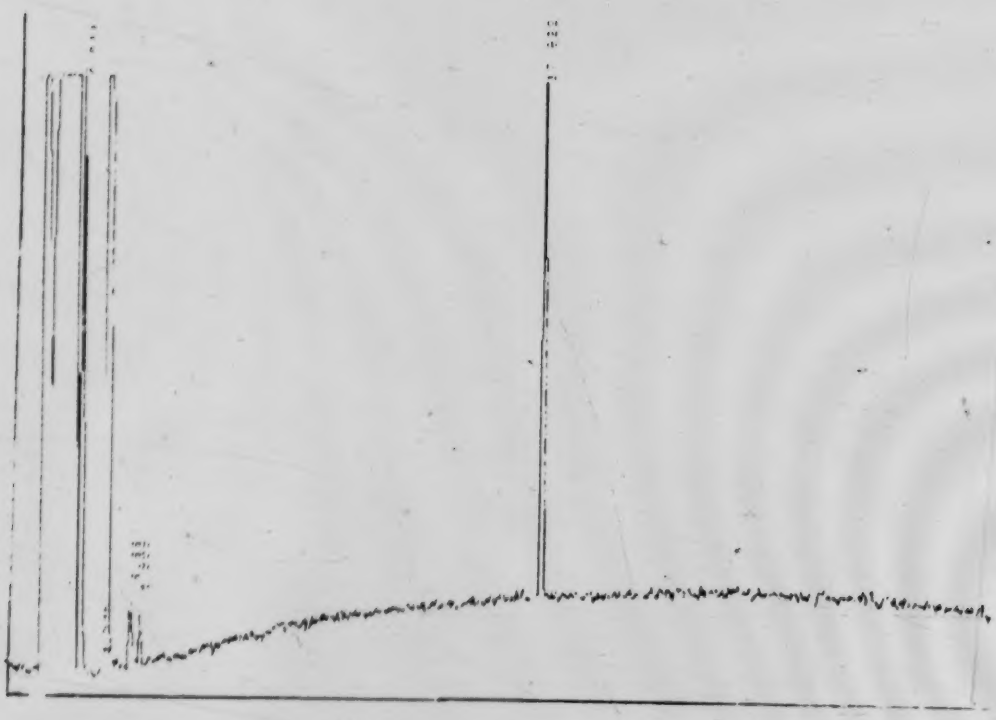


Figure 23. Chromatogram of flesh from fish exposed to dibenzothiophene

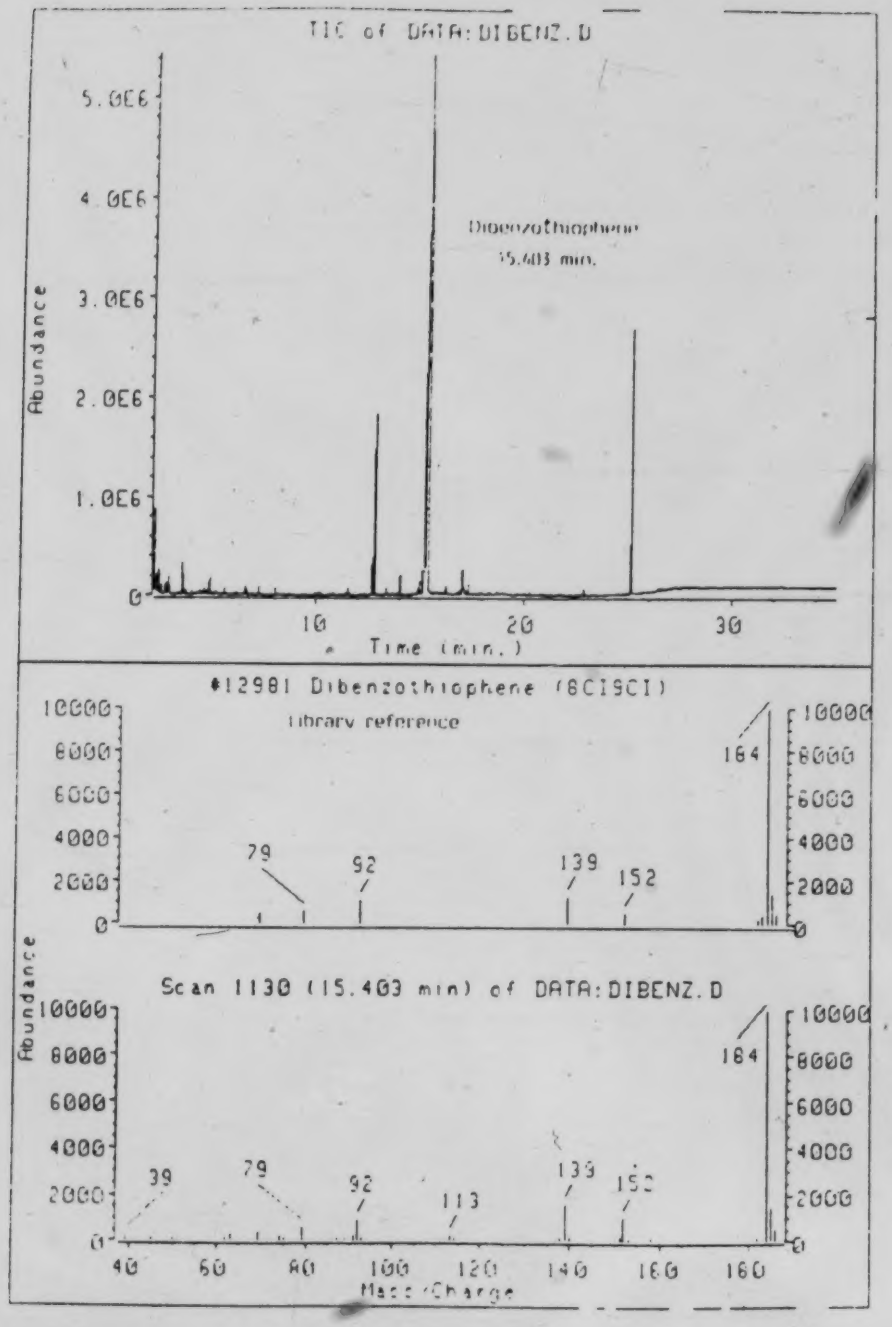


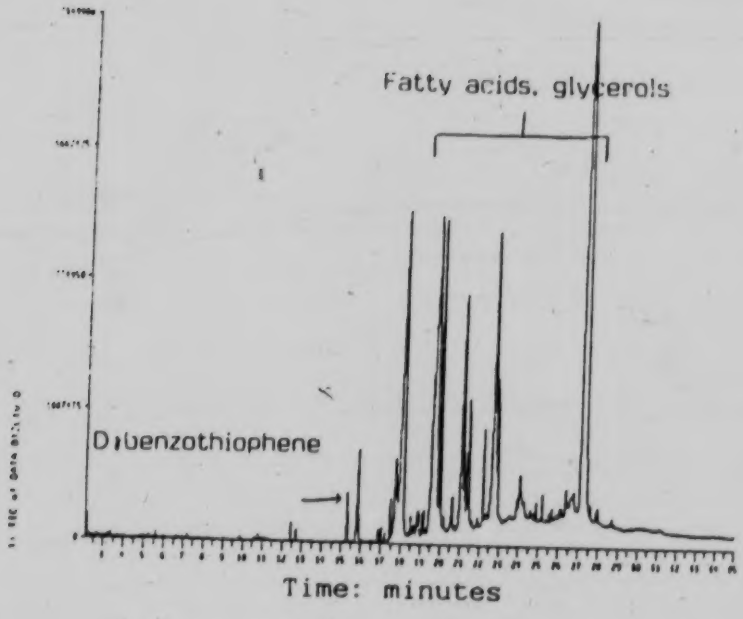
Figure 24. Total ion chromatogram of dibenzothiophene fish flesh.

Although rainbow trout can readily accumulate dibenzothiophene in muscle and liver with an average of 23 and 18.5 µg/g respectively, they are slow to depurate the same compound. After 65h of depuration the fish contained an average of 10.1 and 14.3 µg/g in muscle and liver tissue respectively. These levels of chemical are quite high, considering the fish were in fresh water for 65 h. Such high levels of the compound following depuration, indicate the fish had some difficulty mobilizing dibenzothiophene . Dibenzothiophene appears to be more persistent than benzothiophene in the tissues of salmonids.

4.4.2 Metabolism studies: dibenzothiophene:

Liver and bile samples from fish exposed to dibenzothiophene were examined for the presence of metabolites. Neither primary nor secondary metabolites were found. However, in the case of one depurated liver, there were a number of unidentified peaks which appeared on the GC/FPD and GC/FID chromatograms, approximately 4 minutes after the parent compound peak (Figure 25). This particular extract was then run on GC/MS, in order to try and identify these peaks. However, the relevant spectra did not match any reference library compounds. The extract was then run (with the help of the staff chemist), on a high resolution mass spectrometer (VG Model 7070) and the spectra were determined to be fatty acids and glycerols. There was no evidence of metabolites.

On analysis of bile samples, neither parent compound nor metabolites were found. While the fish were in fresh water for depuration studies, they were fed (and observed to eat) pellet food. We can assume therefore, that digestion occurred, resulting in production and flow of bile. As such, it is surprising that no detectable levels of dibenzothiophene



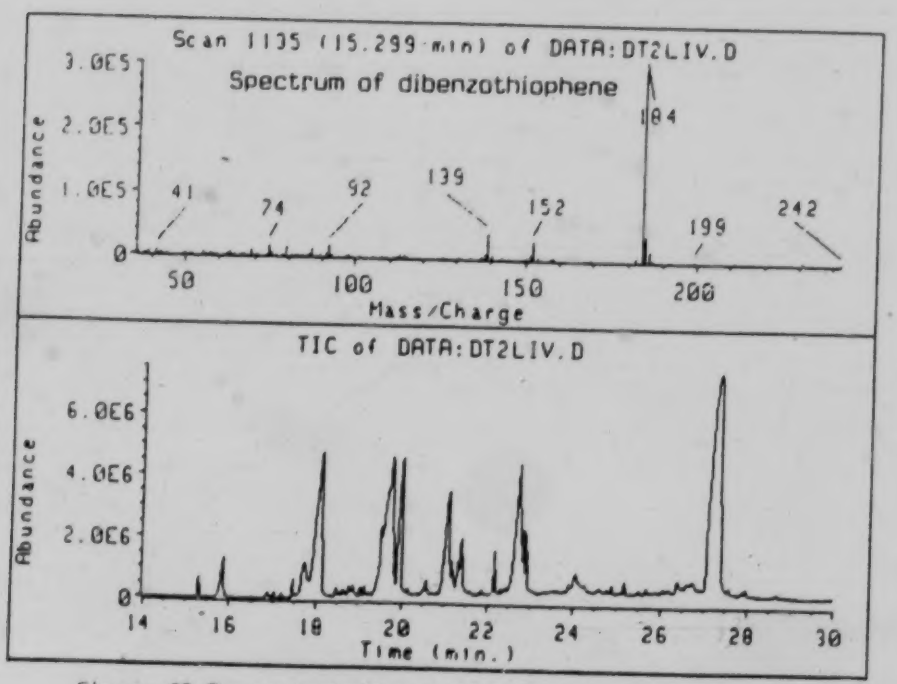


Figure 25 Depurated dibenzothiophene liver. G.C./M.S. chromatogram of extract with identification of parent compound.

were found in the bile, given that the related compound, benzothiophene, was found in ample quantities in the bile of similarly exposed fish.

Dibenzothiophene may be metabolized, but no evidence of this was found. In comparison to benzothiophene, dibenzothiophene is accumulated to a similar extent in both liver and muscle tissue, but is eliminated more slowly. Following depuration in both cases, the muscle contained an average of 2.9 μg/g in the case of benzothiophene and 10.2 μg/g in the case of dibenzothiophene. It appears that fish can eliminate benzothiophene more rapidly than they can dibenzothiophene. This may result from dibenzothiophene having a higher octanol-water partition coefficient (more lipophilic) than benzothiophene. However this is unlikely an explanation for the "all or nothing" results obtained. The reason is more likely to be an analytical problem in finding the metabolites.

4.5 Bioconcentration of benzothiophene and dibenzothiophene.

Bioconcentration factors (BCF) for benzothiophene and dibenzothiophene in fish tissues are reported in Table 11. Both chemicals are bioconcentrated to a similar extent in the muscle and liver of rainbow trout. However there was no evidence of dibenzothiophene being bioconcentrated in the bile, whereas benzothiophene exhibited a mean BCF of 9. One possible explanation for this is that the fish may have had more difficulty in mobilizing dibenzothiophene in the liver and subsequently depositing it in the bile, than they did with benzothiophene. Alternatively, analytical techniques for the recovery of PASH from fish tissues may be lacking and require further development.

The results reported in Table 12 were used to determine additional BCFs of benzothiophene. BCFs in this flesh ranged from 10 to 500. With such a scatter of

Table 12. Tissue concentrations for fish exposed to benzothiophene.

*Exposure Concentration (mg/L)**	*Flesh Concentration (mg/Kg) **
0.01	0.01
0.03	3
0.08	47
0.5	62
1.0	84

* This value is based on the amount of chemical added to the water.

** The 15 samples from each concentration were pooled together by means of a Waring blender. Analyses were performed on these pooled samples using GC/FID.

Table 12a. Exposed flesh as prepared for sensory evaluation panel.

Flesh Concentration (mg/Kg) *
0.01
0.043
0.092
0.54
1.04

* The exposed flesh was mixed with control flesh in order to obtain the desired concentration.

Table 12b. Tissue concentration for fish fortified with benzothiophene. This flesh was fortified for the purpose of sensory evaluation only.

Target Fortification Level (mg/Kg)	*Flesh Concentration (mg/Kg)* *
0.01	0.021
0.03	0.034
0.08	0.086
0.5	0.61
1.0	1.08

BCFs, it is difficult to predict water levels that will produce related concentrations of the chemical in fish.

4.6 Sensory Evaluation:

4.6.1 Taste threshold:

Individual taste and odour thresholds were determined by panelists who made a judgement as to the least detectable concentration of benzothiophene that they could recognize correctly (Jellinek, 1985). Correct responses are taken to be those, above which panelists do not mistakenly identify the controls as having a taint. There were three controls interspersed among the eight pairs.

The sensory threshold determination was carried out with rigorous environmental and procedural controls as outlined in ASTM (1979). The median taste/odour threshold was determined as the lowest concentration where >50% of the population detected a correct response and was found to be 0.01 ppm. The geometric mean odour thresholds were calculated for each panelist. Table 13 gives the individual, individual mean and group mean threshold values. There was no distinction made between taste and odour thresholds, as it has been demonstrated that these two senses are inter-related. The combination of the two senses was used for the sensory evaluations.

For each replicate evaluation, bar charts were constructed depicting the % response of exposed or fortified versus controls at each of the three pairs where a control was included. These charts demonstrate that >50% of the panelists pick exposed or spiked over controls at all values, with the percentage increasing for higher concentrations. The odour

Table 13: Individual, individual mean and group mean thresholds (all calculated means are geometric).

Panelist	*Replicate Analysis*			*Mean Individual Threshold (Geometric Mean)*
	1	*2*	*3*	*(ppm)*
	Individual thresholds (ppm)			
1	0.01	0.03	1.0	0.067
2	0.01	0.01	0.01	0.01
3	0.01	0.08	0.01	0.02
4	0.01	0.01	0.01	0.01
5	0.01	0.01	0.01	0.01
6	0.01	0.08	0.08	0.04
7	0.5	0.03	0.5	0.20
8	0.01	0.01	0.01	0.01
9	0.01	0.01	0.01	0.01
10	0.03	0.5	0.01	0.05
11	0.03	0.01	0.08	0.03
Geometric Group mean	0.02	0.03	0.03	0.04*
				0.02**

* Total Group Mean Threshold

** Recalculated group mean

threshold was thus determined to be 0.01 mg/kg. These charts are depicted in Figures 26 to 28.

The individual mean thresholds ranged from 0.01 mg/kg to 0.20 mg/kg (geometric means, refer to Table 13). Note the large differences between these values for individual panelists. There are three judges in particular who deviate considerably from the rest of the group. On checking the raw data it was noticed that these particular panelists repeatedly identified the controls as having an "off-flavour" and were inconsistent from session to session. Thresholds obtained from these panelists in each of the three replicates were plotted (Figures 29 and 30) and compared with their geometric means. Each of the three exhibited at least one replication greater than four times or less than one quarter times (>4X or <1/4X) his/her geometric mean. According to Rosen *et. al* (1962) and Hamilton *et. al* (1978) a given panelist should distinguish concentrations which differ by a factor of two (2X or >1/2X); it was our decision to use twice their factor of two as a safety measure. This is sufficient criterion for rejecting panelists. Consequently the results of the particular panelists in question were ignored.

A new group mean threshold was calculated based on the remaining 8 panelists. This new group mean threshold was determined to be 0.02 mg/kg as opposed to the old value of 0.04 mg/kg. The geometric mean threshold value of 0.02 mg/kg is more in keeping with the median threshold value of 0.01 mg/kg. Since 0.01 mg/kg is the lowest concentration presented to the panelists, we can safely assume that the actual threshold concentration for benzothiophene is 0.01 mg/kg or less.

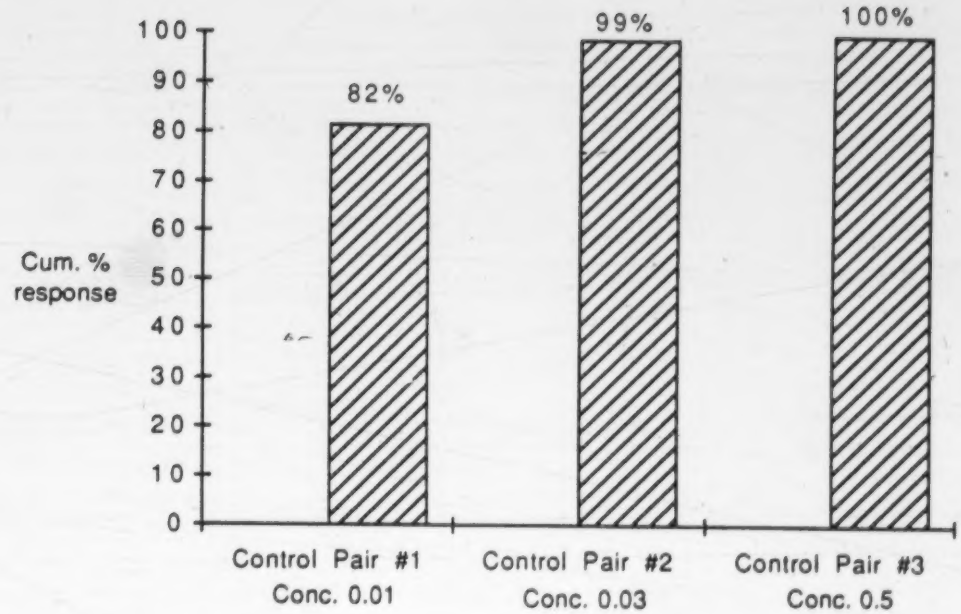


Figure 26. Sensory evaluation, replicate #1, cumulative percent response of exposed or fortified flesh vs control at each of three concentrations.

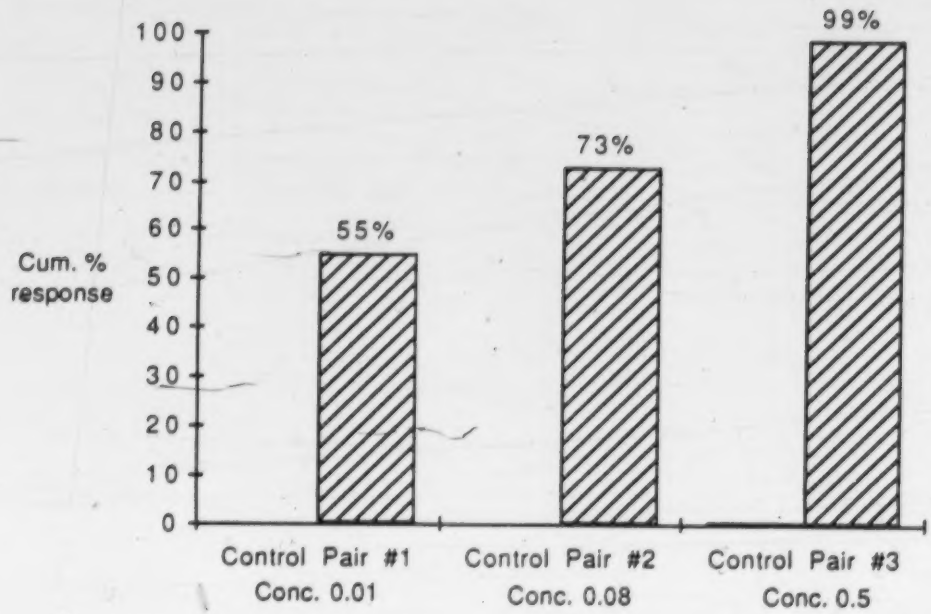


Figure 27. Sensory evaluation, replicate #2, cumulative percent response of exposed or fortified flesh vs control at each of three concentrations.

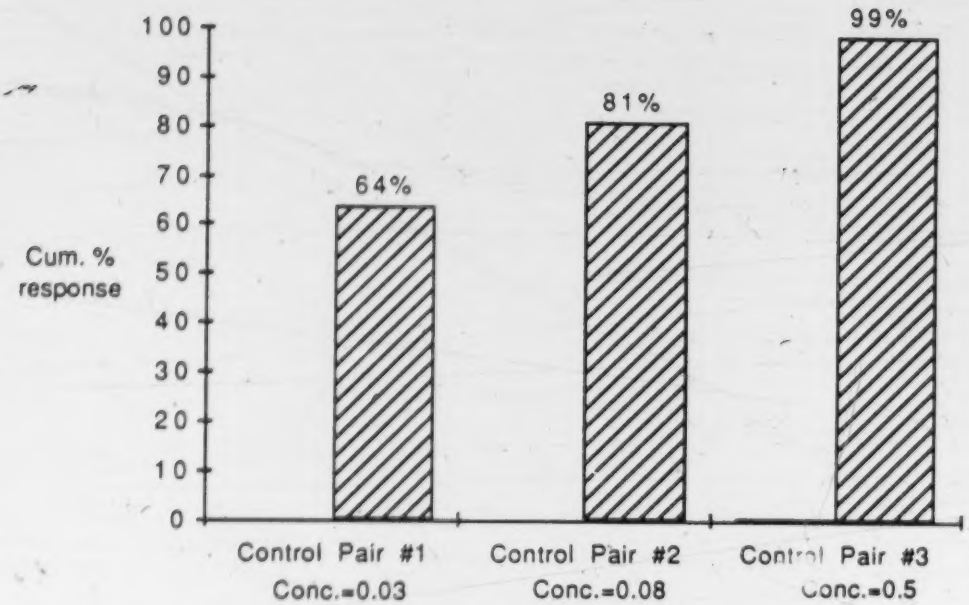


Figure 28. Sensory evaluation, replicate #3, cumulative percent response of exposed or fortified flesh vs control at each of three concentrations.

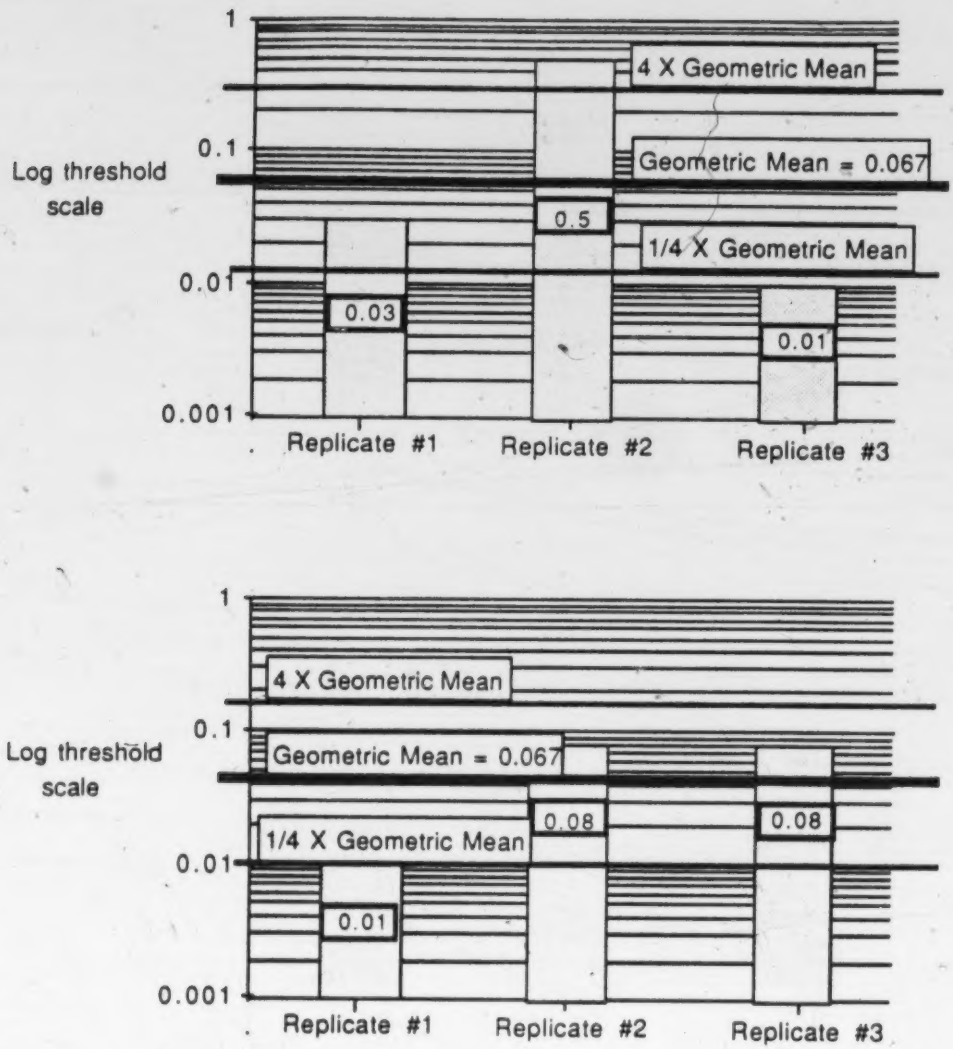
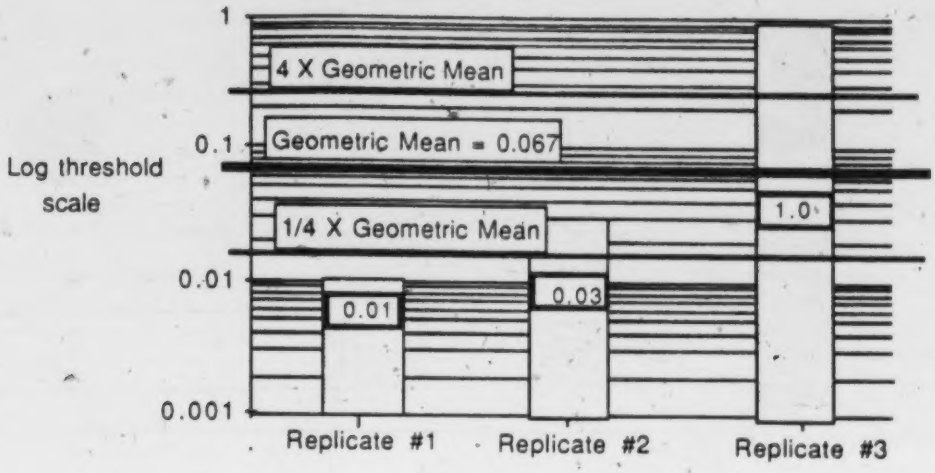


Figure 29. Panelists 6 and 10: plots of individual mean thresholds vs. geometric mean threshold.



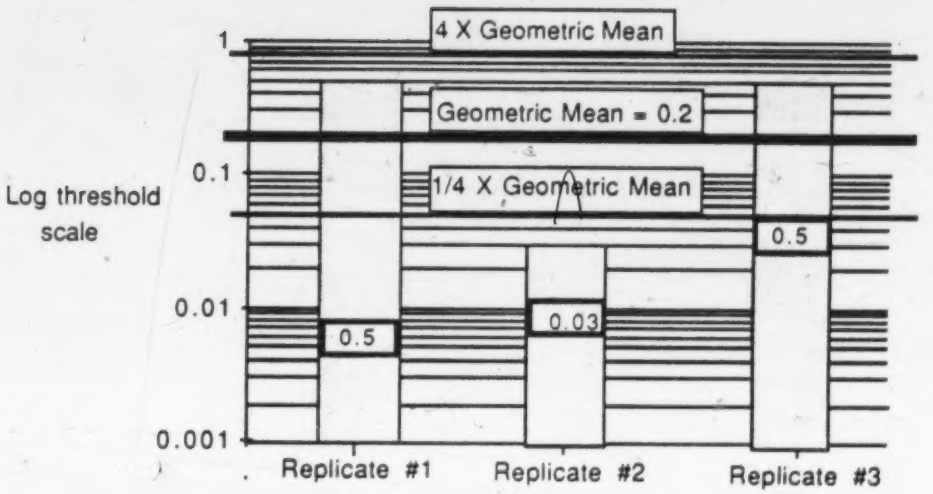


Figure 30. Panelists 1 and 7: plots of individual mean thresholds vs. geometric mean threshold.

4.6.2 Degree of Taint:

Panelists were asked to assign a "degree of taint" on the boxed line provided,to each sample which they choose as being tainted. Panelists were asked to record their response to each sample by placing a mark on the line at a point corresponding to their perceived intensity of the off-flavour in the sample. These values were later converted to numerical scores and are presented in Table 14. Each mean intensity represents a subset of the total number of observations for that treatment, including only individuals who exhibited a threshold odour level below the concentration being judged. Intensities were determined in this manner on the presumption that intensity values assigned for samples which are below each individual's threshold detection level are, by definition not detected and they therefore represent an "incorrect" assignment.

Mean intensities of fortified, exposed and tainted fish are supplied in Table 14. Intensities do not increase proportionally with concentration, as would be expected in such a case. This may be explained by the "saturation effect" (Windholz *et. al*, 1983). At higher concentrations the olfactory senses may be fatigued and fail to perceive increasing concentrations. However, if we do look at the difference in intensities between exposed and spiked samples, we notice an increase in the difference with increasing concentrations. This observation indicates that panelists were more definite in their choice of exposed over fortified at higher concentrations. This observation may be seen more clearly in a graphical representation of the results, provided in Figure 31.

Table 14. Mean intensity scores of fortified and exposed fish; determined from three replicate sensory evaluations using 11 panelists.

Number of Panelists*	Samples	Mean Intensity	Range	
17	E 0.01 ppm	2.3	0-3	E=Exposed fish flesh
5	F 0.01 ppm	0.52	0-7	F=Fortified fish flesh
21	E 0.03 ppm	1.57	0-6	ppm=parts per million
11	F 0.03 ppm	0.83	0-6	
26	E 0.08 ppm	2.67	0-3	Each mean intensity represents
3	F 0.08 ppm	0.18	0-8	a subset of the total number of observations for that treatment
30	E 0.50 ppm	4.03	0-5	including only individuals who
2	F 0.50 ppm	0.24	0-9	exhibited a threshold odour level below the concentration being
33	E 1.00 ppm	0.82	0-9	judged.
0	F 1.00 ppm	-	-	

* There are a total of 33 panelists, based on 11 panelists with 3 replicates.

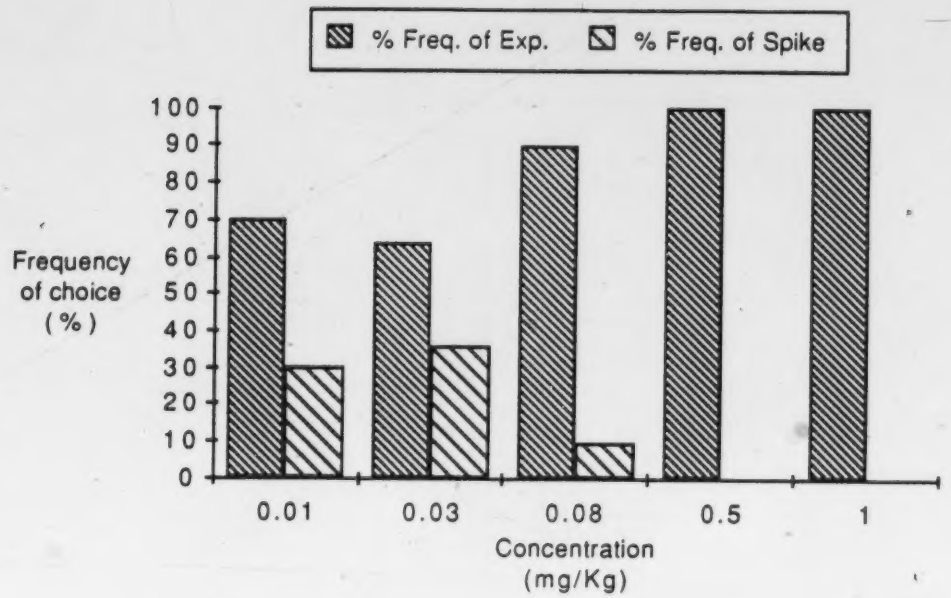


Figure 31. Taste and odour evaluation: average of three replicates.

4.6.3 The Tainted Sample:

The results obtained from the sensory evaluations indicate that the exposed samples are perceived as being more tainted than the fortified samples. This can be seen at a glance in Table 14. The panelists consistently chose the exposed sample as opposed to the fortified, indicated by the large difference in intensities between both the samples. Thus, the spiked approach is conservative as a measure of sensory threshold levels in real life. However the raw data was analyzed for significance of variance between exposed and fortified flesh. From the results of the analysis of variance (ANOVA) presented in Table 15, significant interactions are clearly demonstrated for the taste and odour data. Interactions among concentrations, among taint methods and between concentrations and taint methods were significant at the 1% significance level.

These results can be interpreted as follows:

(1) A significant difference ($F= 17.9$, $P<0.01$) existed between concentration levels and the associated intensity value. The intensity scores increased with increasing concentration.

(2) There was a highly significant difference ($F=265.4$, $P<0.01$) in the selection of taint methods, with exposed flesh being easily favoured over fortified at all concentration levels.

(3) There was a significant interaction ($F=29.8$, $P<0.01$) between the selection of exposed or fortified flesh and the concentration being presented. The frequencies of selections favouring exposed flesh increased with increasing concentration.

In Table 16 an analysis of variance is computed using all perceived intensities, including those below the threshold levels. The results are virtually the same as those

obtained using he perceived intensities above the threshold levels only . The intensity values assigned to the samples by the panelists are provided in Table 17, including those values assigned above the threshold levels only.

Table 15. Analysis of Variance 1: computed perceived intensities above threshold levels only.

	A_1 0.01	A_2 0.03	A_3 0.08	A_4 0.5	A_5 1.0	TOTALS
(Exp.) B_1	Σx=76 Σx^2=412 μ=2.30	Σx=52 Σx^2=222 μ=1.57	Σx=88 Σx^2=376 μ=2.67	Σx=133 Σx^2=675 μ=4.03	Σx=225 Σx^2=1657 μ=6.82	ΣB_1=574 ΣB_1^2=3342 μB_1=3.48
(Fort.) B_2	Σx=17 Σx^2=69 μ=0.52	Σx=27 Σx^2=109 μ=0.82	Σx=6 Σx^2=14 μ=0.18	Σx=8 Σx^2=34 μ=0.24	Σx=0 Σx^2=0 μ=0	ΣB_2=58 ΣB_2^2=226 μB_2=0.35
TOTALS	ΣA_1=93 ΣA_1^2=481 μA_1=1.41	ΣA_2=79 ΣA_2^2=331 μA_2=1.20	ΣA_3=94 ΣA_3^2=390 μA_3=1.42	ΣA_4=141 ΣA_4^2=709 μA_4=2.13	ΣA_4=225 ΣA_4^2=1657 μA_4=3.41	Σx_{tot}=632 Σx_{tot}^2=3568 μ_{tot}=1.92

$SS_{tot} = \Sigma x_{tot}^2 - (\Sigma \hat{A} x_{tot})^2/N_{tot} = 3568-(632)^2/330=3568-1210.37$

$= \underline{2357.63}$

$SS_{bg} = \Sigma[(\Sigma x_g)^2/N_g] - (\Sigma x_{tot})^2/N_{tot} = 8.76+22.1+1.09+1.93+$
$+175.03+81.94+234.7+536.0+1534-1210.37$

$=\underline{1385.18}$

$SS_{conc.} = (\Sigma x_{A1})^2/N_{A1} + (\Sigma x_{A2})^2/N_{A2} + (\Sigma x_{A3})^2/N_{A3} + (\Sigma x_{A4})^2/N_{A4} + (\Sigma x_{A5})^2/N_{A5}$
$(\Sigma x_{tot})^2/N_{tot} = 131.04+94.56+133.88+301.23+767.04-1210.37$

$=\underline{217.37}$

$SS_{taint.} = (\Sigma x_{B1})^2/N_{B1} + (\Sigma x_{B2})^2/N_{B2} - (\Sigma x_{tot})^2/N_{tot} = 1996.82+20.39-1210.37$

$=\underline{806.83}$

$SS_{AB} = 33[(0.4489)+(1.4161)+ (0.0961)+(0.1156)+(3.4225)+(0.4624)+(1.4161)$
$+(0.1089)+ (0.1024)+(3.3856)$

$=\underline{362.16}$

$SS_{wg} = \Sigma[\Sigma x_g^2 - (\Sigma x_g)^2/N_g] = 60.24+86.9+12.91+32.07+236.97+140.06+141.3$
$+139.0+123.0$

$=\underline{972.45}$

ANOVA: Summary table for taste-test

Source	df	SS	MS	F	P
$A_{conc.}$	4	217.37	54.34	17.88	<.01
$B_{taint.}$	1	806.83	806.83	265.40	<.01
AXB	4	362.16	90.54	29.78	<.01
Within groups	320	972.45	3.04		
Total	329	2357.63			

Conclusions of Summary table:

(1) Panelists detected differences in taint between concentrations (increasing).
(2) Panelists detected a taint difference between exposed and fortified flesh.
(3) Panelists detected a greater taint in exposed vs spiked in all cases.

Assuming consistant variability between replicates and judges.

Table 16. Analysis of Variance 2: computed using all perceived intensities, including those below the threshold levels.

	A_1 0.01	A_2 0.03	A_3 0.08	A_4 0.5	A_5 1.0	TOTALS
(Exp.) B_1	Σx=104 Σx^2=540 μ=3.15	Σx=74 Σx^2=314 μ=2.24	Σx=97 Σx^2=405 μ=2.94	Σx=137 Σx^2=691 μ=4.15	Σx=225 Σx^2=1657 μ=6.82	ΣB_1=637 ΣB_1^2=3607 μB_1=3.8
(Fort.) B_2	Σx=23 Σx^2=87 μ=0.70	Σx=30 Σx^2=118 μ=0.91	Σx=7 Σx^2=15 μ=0.21	Σx=8 Σx^2=34 μ=0.24	Σx=0 Σx^2=0 μ=0	ΣB_2=68 ΣB_2^2=254 μB_2=0.41
TOTALS	ΣA_1=127 ΣA_1^2=627 μA_1=1.92	ΣA_2=104 ΣA_2^2=432 μA_2=1.38	ΣA_3=104 ΣA_3^2=420 μA_3=1.58	ΣA_4=145 ΣA_4^2=725 μA_4=2.20	ΣA_4=225 ΣA_4^2=1657 μA_4=3.41	Σx_{tot}=705 Σx_{tot}^2=3861 μ_{tot}=2.14

$SS_{tot} = \Sigma x_{tot}^2 - (\Sigma x_{tot})^2/N_{tot} = 3861-(705)^2/330 = 3861-1506.14$

$= \underline{2354.9}$

$SS_{bg} = \Sigma[(\Sigma x_g)^2/N_g] - (\Sigma x_{tot})^2/N_{tot} = 327.8+165.9+285.1+586.6+1534.1+1608+$
$+273+1.48+1.94+0-1506.14$

$=\underline{1422.1}$

$SS_{conc.} = (\Sigma x_{A1})^2/N_{A1} + (\Sigma x_{A2})^2/N_{A2} + (\Sigma x_{A3})^2/N_{A3} + (\Sigma x_{A4})^2/N_{A4} + (\Sigma x_{A5})^2/N_{A5}$
$(\Sigma x_{tot})^2/N_{tot} = 244.38+163.88+318.56+767.04-1506.14$

$=\underline{151.6}$

$SS_{taint.} = (\Sigma x_{B1})^2/N_{B1} + (\Sigma x_{B2})^2/N_{B2} - (\Sigma x_{tot})^2/N_{tot} = 2459.21+28.02-1506.14$

$=\underline{981.1}$

$SS_{AB} = 33[(0.24)+(1.12)+ (0.13)+(0.05)+(2.86)+(0.26)+(1.12)$
$+(0.13)+ (0.05)+(2.83)$

$=\underline{290.1}$

$SS_{wg} = \Sigma[\Sigma x_g^2 - (\Sigma x_g)^2/N_g] = 212.2+148.1+119.9+122.4+122.9+71+90.7$
$+13.5+32.06$

$=\underline{932.8}$

ANOVA: Summary table for taste-test

Source	df	SS	MS	F	P
$A_{conc.}$	4	151.6	37.9	13.07	<.01
$B_{taint.}$	1	981.1	981.1	338.31	<.01
AXB	4	290.1	72.5	25.0	<.01
Within groups	320	932.8	2.90		
Total	329	2355.6			

Conclusions of Summary table: As in Table 15.

(1) Panelists detected differences in taint between concentrations (increasing).
(2) Panelists detected a taint difference between exposed and fortified flesh.
(3) Panelists detected a greater taint in exposed vs spiked in all cases.

Assuming consistant variability between replicates and panelists.

Table 17. Intensity values assigned to samples including percieved intensities above the threshold levels only.

Judge	Rep.	{Concentrations (mg/kg): Exposed (E) vs Fortified (F)}									
#	#	.01E	.01F	.03E	.03F	.08E	.08F	.5E	.5F	1.0E	1.0F
1	*1*	0	4	0	6	2	0	4	0	5	0	0
	2	0	0	0	1	0	1	2	0	1	0	2
	3	0	0	0	0	0	0	0	0	8	0	0
2	*1*	7	0	0	1	6	0	9	0	5	0	0
	2	7	0	4	0	2	0	7	0	9	0	0
	3	3	0	3	0	4	0	0	5	7	0	0
3	*1*	7	0	3	0	3	0	5	0	7	0	0
	2	0	0	0	0	3	0	0	3	7	0	0
	3	0	3	0	4	4	0	4	0	6	0	0
4	*1*	5	0	4	0	4	0	4	0	9	0	0
	2	7	0	0	1	1	0	5	0	4	0	0
	3	6	0	2	0	0	3	4	0	4	0	0
5	*1*	4	0	0	1	1	0	3	0	4	0	0
	2	2	0	0	1	3	0	3	0	3	0	0
	3	2	0	0	1	2	0	4	0	7	0	0
6	*1*	0	6	0	5	2	0	7	0	8	0	0
	2	0	0	0	0	0	2	3	0	9	0	0
	3	0	0	0	0	3	0	7	0	8	0	3
7	*1*	0	0	0	0	0	0	6	0	8	0	0
	2	0	0	2	0	7	0	3	0	8	0	2
	3	0	0	0	0	0	0	2	0	5	0	5
8	*1*	0	2	3	0	2	0	4	0	9	0	0
	2	2	0	6	0	2	0	5	0	9	0	0
	3	0	2	0	5	5	0	4	0	8	0	0
9	*1*	7	0	4	0	5	0	4	0	6	0	0
	2	3	0	0	1	4	0	5	0	8	0	0
	3	5	0	4	0	4	0	4	0	8	0	0
10	*1*	0	0	6	0	3	0	6	0	7	0	5
	2	0	0	0	0	0	0	1	0	7	0	0
	3	1	0	5	0	3	0	3	0	7	0	0
11	*1*	5	0	5	0	8	0	6	0	8	0	5
	2	3	0	1	0	4	0	5	0	8	0	0
	3	0	0	0	0	1	0	4	0	8	0	1

5. Discussion

5.1 Methodology:

The simulation of environmental exposure is perhaps the most difficult parameter to incorporate in laboratory tests. However, the use of this particular laboratory study, as opposed to a field study, has several inherent advantages: both genetically and physiologically homogenous material may be chosen; the physiological condition of animals before exposures can be controlled in respect to their nutritional and thermal history; the exact time and duration of the exposure may be known and the chemical concentrations can be closely monitored.

Complete sample homogenization is essential when accurately quantitating xenobiotics in biological samples, especially fish, where the fat is heterogenously distributed. Benville and Tindle (1970) found the dry ice procedure kept the frozen tissue brittle and vulnerable to fragmentation. In this study the dry-ice procedure was found to be effective and efficient, yielding an homogenous sample in the minimum of time.

Solvent extraction was a necessary step in the recovery of the contaminant from the fish tissue. It was found that 280 mL of solvent (methylene chloride) was needed to extract a 20 g sample of fish, mixed with 80 g of anhydrous sodium sulphate. A minimum of 4 h soxhlet extraction was required to achieve >90% recovery. Less time than this yielded poor recovery. In this particular study, a minimum of 5 h soxhlet extraction was observed.

Stalling *et al.* (1972) reported greater than 95% recovery using gel permeation chromatography. Recovery of benzothiophene was 97 +/- 2% and that of

dibenzothiophene was 95 +/- 2%. Rainbow trout have a high lipid content (7-10%) and as such are difficult to clean. However, gel permeation chromatography was found to effectively remove the lipid content of the tissue extract. G.P.C. is unique in that separations are based primarily on differences in molecular size and since molecular weight of most lipids are between 600 and 1500 and those of benzothiophene and dibenzothiophene are between 130 and 200, G.P.C. can effectively separate the lipids from the xenobiotics.

Stalling *et al.* (1972) found bio-beads to be superior to Sephadex in gel chromatography. Since clean-up of the fish extract was essential in our analysis, bio-beads SX3 were chosen in preference to Sephadex. Additional clean-up with florisil is recommended for samples in which whole body PAH residues are less than 0.1 μg/g (Stalling *et al.*, 1972) and since we were unsure as to the quantity of xenobiotics in the fish tissue, florisil cleanup was chosen as an added precaution. The results demonstrate that whole body concentrations greatly exceeded this value, however.

5.2 Accumulation Studies:

There are at least two paths by which fish can take up hydrocarbon (Lee *et .al*, 1972 and 1976; Varanasi *et. al*, 1979; Roubal *et. al*, 1977 and Sanborn and Malins, 1977). The first path is with the food, where hydrocarbon entrance is via the gut. The second path is dealt with in this report, namely, uptake of hydrocarbon dissolved in the water, where entrance is through the gills. Varanasi *et al.* (1978) reported a third path of uptake, the skin and demonstrated that the skin of rainbow trout is actively involved in the uptake and discharge of hydrocarbons. In general, however, the literature suggests that the gills are the primary route of uptake.

Lee, *et al.* (1972) suggest that the gills have a micellar layer which adsorbs the hydrocarbons and then passes them on to other tissues. Stein, *et al.* (1984) concur with the theory that the gills are a significant non-dietary route of uptake of hydrocarbons, but suggest that they are not a major route of excretion. McKim and Heath (1983) have shown that 76% of a hydrocarbon in inspired water is adsorbed in one pass, by the gills of two trout species and that negligible amounts are excreted via the gills.

Once absorbed through the gills, the hydrocarbons are then distributed into the blood system and transported via the arterial system to the tissues and organs throughout the body. Most literature reports that the hydrocarbons then build up in the liver, where metabolism first occurs (Lee, *et al.*, 1972; Varanasi and Gmur, 1980 & 1981; Stein *et al.*, 1984 and Thomas and Rice, 1982) . In studies with naphthalene, it has been shown that the majority of this compound taken up by the fish is metabolized in the liver and excreted into the bile. There appears to have been no such studies with benzothiophene and dibenzothiophene. In the absence of any other information, the documented route for the hydrocarbons should be considered possible routes for the two PASH compounds.

The rapid uptake of benzothiophene and dibenzothiophene by rainbow trout was similar to results obtained using naphthalene and phenanthrene as hydrocarbons of interest (Eastmond *et. al*,1984). Both PASH were readily taken up and bioaccumulated in the muscle and liver of rainbow trout. As compared to dibenzothiophene, benzothiophene was taken up more rapidly; accumulated to higher levels in all tissues studied; stored in the liver to a much greater extent as parent compound; and was eliminated more rapidly from muscle and liver tissue.

There was no accumulation of dibenzothiophene in the bile studied. Absence from the bile might indicate rapid metabolism by the mixed-function oxidase (MFO) enzyme

system, as indicated by Stegeman (1981). However, there was no evidence of conjugated or non-conjugated metabolites in the bile studied. In the case of benzothiophene, the liver was found to have the highest concentration , whereas muscle exhibits the highest concentration in the case of dibenzothiophene.

5.3 Elimination studies:

There are two routes of excretion presented in the literature; renal and bilary, with bile appearing to be the major route. Previous studies (Varanasi *et al,* 1982a & 1982b) have indicated that both parent compound and metabolites are deposited in the bile juices. In the case of benzothiophene (no depuration) parent compound was found in the bile juices, but, there was no evidence of parent compound in bile of fish exposed to dibenzothiophene.

Cravedi and Tulliez (1982), suggested that starvation has an adverse effect on rainbow trout for depuration of hydrocarbons. They noticed that the rate of mobilization of stored hydrocarbons by animals fed an energy-restricted diet was less than that of those receiving control diet. They concluded that in aquatic organisms, mobilization of foreign compounds occurred very slowly, especially during starvation. This might be partly due to a slow lipid utilization resulting from a low metabolic rate during starvation. Because of these observations, fish used in depuration studies in this experiment were fed for the duration. Although the fish were reluctant to eat the food in the first 8 h of being in clean water, they commenced eating soon thereafter. All gall bladders expirated for analysis were full, indicating that digestion and mobilization was in process.

The reason why benzothiophene is eliminated from the tissues to a much greater extent than dibenzothiophene is unclear. Possibly as Lee, *et al.* (1972) suggested in the

case of benzo(a)pyrene, dibenzothiophene causes a diminution of the metabolic rate, so that less hydrocarbon is processed and eliminated. This may also explain why less dibenzothiophene is taken up by the fish as compared to benzothiophene. When evaluating the persistence of chemicals in fish, we are instructed by Lech and Bend (1980) to consider that multiple mechanisms of elimination may exist and while metabolism is important for certain compounds, alternate mechanisms may predominate for others. In studies with rainbow trout they found that certain hydrocarbons are metabolized very little, if at all, but can be eliminated gradually over a period of time through the egg mass in the case of the female and the sperm in the case of the male. It may be expected therefore, that such alternate mechanisms are important for compounds that are metabolized slowly *in vivo*. Such may be the situation with dibenzothiophene.

Dibenzothiophene is more persistent than benzothiophene, clearly indicated by the high levels remaining in the liver after depuration. Benzothiophene, on the other hand, decreased by as much as ten fold in depurated livers. Obviously the fish have less trouble eliminating benzothiophene from the tissues, than they do dibenzothiophene.

5.4 Metabolism Studies:

Vertebrates, including fish are able to metabolize various types of hydrocarbons, as outlined by: Diamond and Clark, (1970); Clark and Diamond (1971); Lee *et al.*, (1972); Varanasi and Gmur (1981); Varanasi, *et al.* (1982b); Krahn and Malins (1982) and Krahn *et al.*, (1984). There are numerous papers reporting the metabolism of hydrocarbons such as naphthalene , however, there have been no reports on the metabolism sulphur hydrocarbons such as benzothiophene and dibenzothiophene.

Polycyclic aromatic hydrocarbons are metabolized in the liver by a small number of reactions mediated by enzymes (Kappas and Alvares, 1975): oxidations, reductions, hyrolysis and conjugations. It is known that the introduction into fish of lipid soluble hydrocarbon causes activation of these various enzyme systems (Lee *et al.*, 1972; Sims, 1967). Their essential effect is to convert lipophilic compounds like benzothiophene and dibenzothiophene into hydrophilic ones. These hydrophilic compounds are more easily removed from the blood by the kidneys and excreted.

Initial steps in the bioconversion of aromatic hydrocarbons take place in the endoplasmic reticulum of the cell. The enzymes, aryl hydrocarbon hydroxylases (AHH) operate in unison with the electron transport system of the cell (i.e. via cytochrome P-450) (Roubal, *et al.*, 1978; Malins, *et al.*, 1979, Krahn *et al.* , 1981). Oxygen from the electron transport system combines with the aromatic nucleus to form an epoxide which is subsequently converted to compounds such as mono- and dihydric alcohols (Lech, 1974).

Conjugation is often a second step that comes after metabolism by oxidation, reduction or hydrolysis. Conjugations with natural constituents like glucuronic acid occurs in the presence of the appropriate enzyme. Conjugating enzymes such as glutathione-S-epoxide mediate the additional secondary conversions to conjugate derivatives, which increase the hydrophilic nature of the initial oxidation products. These conjugated derivatives include mercapturic acids, glucuronides, sulphates and glycosides. In my research, the enzyme glucuronidase was provided to remove a possible conjugation product such as glucuronide acid and thus identify the remaining compound, an alcohol, more easily.

Gruger, *et al.* (1977) has previously shown that the AHH of certain salmonids are induced by exposure of the fish to aromatic hydrocarbons, so it is possible that both

benzothiophene and dibenzothiophene induce AHH activity in rainbow trout. The comparatively small amount of benzothiophene found in the liver of depurated fish indicates that the chemical was mobilized in some manner. Although no metabolites were found, it is suspected that benzothiophene was indeed converted to metabolites. A graphical representation of one possible pathway for the metabolism of benzothiophene is illustrated in Figure 21. In this scheme, the benzothiophene is converted initially to the epoxide via the cytochrome P-450 and converted to the monohydric alcohol, followed by an additional reaction of the compound: glucuronic acid is incorporated into the molecule. As such, the metabolite can be more easily discharged through normal renal routes. However, as explained earlier, when the bile extract was enzymatically hydrolyzed with glucuronidase solution, the expected product was not found (i.e. an alcohol). We know that these suspected metabolites are not glucuronides, we also know that they are not phenols, as acetylation of the phenolic compound would have yielded a compound easy to identify with the G.C.

Dibenzothiophene, being more complex, is not readily eliminated; in fact, it accumulates in the liver. It has been demonstrated by Varanasi, *et al.* (1982a) that the major route of excretion of high molecular weight PAH's is via bile in fish. However, in the present study with dibenzothiophene, neither parent compound nor metabolites (conjugated or non-conjugated) were found in the bile and the concentration of dibenzothiophene in the liver did not greatly decrease following depuration. This would seem to indicate that the fish have more difficulty mobilizing dibenzothiophene than they do benzothiophene. This does not automatically lead to the conclusion that rainbow trout cannot metabolize dibenzothiophene. Rather, that it is more persistent. It is possible that there were unidentified metabolites present in the bile. As was the case with benzothiophene, we are assured that these possible metabolites are not glucuronides.

5.6 PASH as petroleum markers:

The present experiment demonstrated that organic sulphur compounds rapidly transfer from the water medium to fish. Since polycyclic aromatic sulphur compounds are contained in crude oil, bitumen and to a lesser extent in refined petroleum products, they may be useful as a marker of oil pollution. Dibenzothiophene, being the more persistent of the two compounds studied would appear to be more suitable as a petroleum marker. The fish can easily accumulate this particular PASH, but have difficulty in its mobilization and elimination.

Previously, researchers have attempted to select compounds contained in crude oil as a marker of oil pollution in fish (Ogata and Miyake, 1973, 1978, Ogata and Ogura, 1976, Krahn *et al.*, 1984 and Oikari and Kunnamo-Ojala, 1987) but no specific compounds were chosen. Ogata and Miyake (1973) suggested that aromatic sulphur compounds be chosen. Although, dibenzothiophene is recommended as a petroleum marker in this particular study, previous studies (Oikari and Kunnamo-Ojala, 1987; Krahn *et al.*, 1984; and Krahn and Malins, 1982) have concluded that metabolites of aromatic hydrocarbons, as opposed to the parent compound, are sensitive and quantitative tools for assessing aquatic contamination.

Krahn *et al.* (1984) suggest that the existence of AHH (aryl hydrocarbon hydroxylase) activity in fish may provide a convenient means of assessing previous exposure to petroleum, or other products containing PAH's. However, not all aromatic hydrocarbons are metabolized in the fish and it is possible that the sulphur hydrocarbon, dibenzothiophene is a similar case. Further research is required to ascertain if this theory is indeed correct. Furthermore, for a better understanding of how the bile patterns of conjugated and non-conjugated metabolites reflect the water quality, more should be known

about bile formation kinetics, not only at constant concentrations, but also at fluctuating ambient concentrations of xenobiotics in the water.

5.7 Tainting potential of benzothiophene:

Heavy pollution and offensive odours may be found in water near petroleum industrial districts and oil spillage. Baldwin *et al.* (1970) and Ogata and Miyake (1973) identified toluene as a possible main cause of the odour. In this study, benzothiophene was identified as an objectionable odour source. Panelists could detect the compound at concentrations as low as 0.01 mg/kg. From previous studies (Ogata and Miyake, 1973) it was concluded that substances imparting an offensive odour to fish have the ability to infiltrate the fish. Benzothiophene, in this study, certainly meets these two criteria. It has been shown in this experiment, that the compound rapidly enters the fish and does indeed impart an offensive odour.

This appears to be the only research of its kind, to date, which involved exposing fish to a known quantity of benzothiophene and performing a sensory analysis on the resulting flesh. Concentrations were always determined by GC prior to sensory evaluation.

The threshold odour concentration (T.O.C.) for benzothiophene was determined to be 0.01 mg/kg or less, as this was the lowest concentration available to the panelists. Persson (1984) defined estimated threshold concentration (E.T.C.) as the lowest concentration of the compound in water that would lead to impairment of the fish flavour. The T.O.C. is that concentration detectable or recognizable in fish by a group of observers. T.O.C.'s of compounds in the flesh of fish are rarely reported and even less (if any) are

reported for fish actually exposed to a specific compound and analysed by a panel of trained judges, as was the case in this experiment.

In practical situations, it may be important to know whether the flavour of fish will be impaired at water concentrations lower than those detectable by the human olfactory system, i.e. whether E.T.C. is less than T.O.C.. Benzothiophene could fall into this category, as this compound is readily accumulated from low concentrations in the water. Theoretically, it is possible, even probable, given the bioconcentration factors supplied in my research, that the water concentration could fail to impart an offensive odour, but an exposed fish could bioconcentrate the compound and exhibit an obnoxious odour. An important observation from this information, is that the aroma of the water is an inadequate indicator of the fish living in it.

Panelists were asked to choose the most tainted sample from a pair of samples: one exposed to benzothiophene, the other fortified with benzothiophene. Both samples had similar concentrations of benzothiophene. In each of three replicates, panelists consistently chose the exposed flesh over the fortified flesh. This is somewhat baffling, considering the concentrations are so similar. One possible explanation is that during uptake of the compound, fish partially convert benzothiophene to some more obnoxious metabolite, whereas in the fortified sample, there is no manner in which this could occur. However, although metabolism of benzothiophene is suspected, no evidence was found to support this suspicion. Another possible explanation is that the exposed sample is more homogenous that the fortified, given that it was distributed to the muscle via the numerous arteries and arterioles, whereas with the fortified sample, the chemical was mixed in with the aid of a blender.

The latter explanation is somewhat dubious, as the sensory results would seem to indicate that the panelists were consistent in not selecting the fortified fish as being more tainted, whereas a non-homogenous sample should lead to inconsistency rather than consistency. A third possibility may be that of additive or synergistic effects. Mann (1982) and Westman and Hoff (1963, cited in Persson, 1984) found that the presence of other compounds in the water greatly influenced the intensity of off-flavours produced by specific compounds. The fish were exposed in dechlorinated tap water, with residual organics removed by an activated carbon column. Therefore, although the "synergistic effect" is interesting, it is unlikely in this instance.

5.8 Panelists

There were three panelists who had difficulty in identifying the tainted samples. They randomLy choose the control sample over the tainted sample. The extent to which these three panelists were inconsistent is graphically demonstrated in Figures 29 and 30. Although these three panelists did not particularly alter the overall sensory results, they do increase the geometric group mean threshold. Including these three judges, the group mean is 0.04 mg/kg, however, when these three judges are left out of the calculation, the group mean is 0.02 mg/kg.

Because of these differences the results are presented in both cases; using only the eight consistent judges and conversely, using all eleven judges. Because one of the criteria in selecting the judges was that they be consistent in their choice of sample, the geometric mean values excluding the outlyers are most useful. The data show that these particular judges were far from consistent in their choices.

6. Summary and conclusions

A series of exposure/depuration experiments with rainbow trout were performed, using benzothiophene and dibenzothiophene as compounds of interest. The results demonstrate that these polycyclic aromatic sulphur heterocycles (PASH) are readily bioconcentrated from waterborne concentrations. Bioconcentration factors (BCF) were determined and ranged from 10 to 500. Benzothiophene is accumulated more rapidly than dibenzothiophene in all tissues studied, with the greatest concentrations being found in the liver.

Following 65h depuration, concentrations of both chemicals were substantially reduced in all tissues examined. However, dibenzothiophene appeared to be more persistent than benzothiophene, with concentrations ten times those of benzothiophene present in the flesh following depuration. This indicates the fish had more difficulty mobilizing dibenzothiophene.

There was no evidence of metabolism with either of the chemicals examined, however, this does not necessarily mean that there were no metabolites. The methods of analysis employed were those commonly used for PAH. Analytical techniques for the identification of sulphur heterocycles have not been fully researched; further studies in this area are required before conclusive results about metabolism can be obtained.

In order to identify substances imparting an offensive odour to fish, trout were exposed to varying concentrations of benzothiophene (0.01 to 1.0 mg/L) and compared for taint with similar concentrations of fortified flesh. The chemical was found to impart an offensive odour to fish flesh, even at concentrations as low as 0.01 mg/kg. The threshold

odour level was determined to be 0.01 mg/kg., but since this was the lowest concentration presented to the panelists, it may be assumed that the actual threshold level is less than this.

Statistical analysis of the data indicate that exposed flesh is significantly more tainted than fortified flesh. The exposed flesh was easily favoured over the fortified flesh at all concentration levels examined. Considering both samples had similar concentrations (confirmed by GC-FID) there are some possible explanations for this observation. The exposed flesh may have been more homogenous as the chemical was distributed via numerous arteries and arterioles, whereas it was manually added to fortified flesh. However, results indicate that this is not the case. Alternatively, the exposed fish may have converted benzothiophene to some more obnoxious metabolite, whereas this could not have occurred in the fortified flesh.

Although the latter explanation is favoured, there was no evidence of metabolism in the exposed flesh. As mentioned earlier, this may be the fault of the analytical techniques employed rather than the inability of the fish to metabolize the chemical.

7. Recommendations

Oil tainting of aquatic organisms has been a concern for numerous years. Unfortunately the lack of sufficient analytical techniques for identifying and quantifying the compounds has lead to continuing confusion about this topic. Procedures for sensory evaluation are varied and ambiguous. Threshold odour levels of a few relevant contaminants have been reported, however, the methods of determining them have been difficult to reconcile with modern analytical techniques.

High sensitivity of the olfactory system to sulphur containing compounds has been reported in the literature, yet there have been few studies reported on PASH and their ability to taint fish flesh. The techniques reported for PASH analysis are sadly lacking - with no studies reported on the metabolism of such compounds.

In order to properly address the problem of oil tainting, certain deficiencies must be corrected:

* Further research is required on elimination and metabolism of petroleum components. In order to properly assess the oil tainting problem a basic understanding of the biodegradation and elimination of the components is necessary.

* There is a need to determine what levels of exposure to the major compound classes are necessary to cause tainting over short and long-term exposure periods.

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APPENDIX 1

Sensory release and waiver form

RELEASE AND WAIVER

THIS AGREEMENT made the day of , 1986.

BETWEEN:

THE UNIVERSITY OF ALBERTA, (hereinafter called "the University") and CINDY JARDINE, EDEL DROMEY, WENDELL KONING, all of the City of Edmonton, in the Province of Alberta, (hereinafter called "the Investigators"),

OF THE FIRST PART,

- and -

of the City of Edmonton, in the Province of Alberta, (hereinafter referred to as "the Subject"),

OF THE SECOND PART,

WHEREAS the Subject has agreed to participate in an experiment whereby the subject will be making odour and/or taste evaluations on fish tainted by hydrocarbon compounds;

AND WHEREAS the Subject adknowledges that the Investigators have informed the Subject of the nature and conditions of said experiment:

NOW THEREFORE this Agreement witnesseth that in pursuance of the said Agreement, and in consideration of the sum of ONE DOLLAR ($1.00) and other good and valuable consideration, the receipt of which is hereby acknowledged, the Subject, his/her heirs, executors, administrators and assigns, do hereby remise release, acquit and forever discharge, to the extent permitted by law, the University, its staff associated with said experiment, and the Investigators, their and each of their respective heirs, executors, administrators, successors and assigns of and from all manner of action or actions, cause or causes of action, suits, dues, sums of money, claims, demands or damages whatsoever, at law or in equity, whch the Subject has had or now has, or otherwise hereafter can, shall or may have, for or by reason of all KNOWN and UNKNOWN injury, loss or damage which has resulted or may result to the Subject from or in respect of or arising out of any matter or thing whatsoever existing up to the present time, and, more particularly, from or in respect of or arising out of said experiment.

AND the Subject, for the consideration aforesaid, hereby covenants and agrees to notify the Investigators and/or the University of any reactions, allergic or otherwise, which the Subject may experience during or following said experiment.

AND the Subject, for the consideration aforesaid hereby covenants and agrees not to make any claim or to take any proceedings on account of any injury, loss or damage against any

person, firm, or corporation who may claim contribution or indemnity from the University or the Investigators.

IT IS A TERM of this Release that the payment of the said money and the passing of the said consideration as aforesaid is not and is not to be deemed in any way to be an admission of liability on the part of any of the parties hereto.

IN WITNESS WHEREOF I have hereunto set my hand and seal this day of , 1986.

SIGNED, SEALED AND DELIVERED)
in the presence of)
)
_____) _____
)

APPENDIX 2

Sensory screening test forms

SCORING OF FISH TAINTING

NAME_____ DATE_____

After evaluating the first pair of samples in the screening test, score the taint of the most tainted sample on the table below. First, record the three-digit code of the most tainted sample, then evaluate the degree of taint of this sample by ticking under the most appropriate category (see "Example" below). Repeat this procedure for each subsequent pair of samples.

Pair No.	Tainted Sample Code	Degree of Taint				
		trace taint	slight taint	moderate taint	pronounced taint	extreme taint
Example	123				✓	
1						
2						
3						
4						
5						
6						
7						

SCREENING TEST FOR TASTE

NAME_____DATE_____

Eight coded samples are provided. Each of these cups contains weak water solutions of chemicals representing the four basic taste sensations. One or more of these may be a "blank" of distilled water, or may be a duplicate sample.

Rinse your mouth with the water provided and take a bite of cracker before tasting each sample. Taste each sample separately and in the order indicated. For each sample, record under "Taste Description" if the sample is tasteless, or has a sweet, salty, sour or bitter taste.

Sample Code Number	Taste Description
451	
234	
171	
200	
678	
328	
448	
107	

SCREENING TEST FOR ODOUR

NAME_____ DATE_____

Ten coded samples are provided. Each of these test tubes contains a dilute solution of a compound having a typical odour

Sniff each sample separately and in the order indicated. Rinse your mouth with the water provided and take a bite of cracker after smelling each sample. Wait approximately 15 seconds between samples. Record your description of each sample under "Odour Description".

Sample Code Number	Odour Description
491	
752	
326	
903	
452	
621	
587	
321	
834	
787	